Co-infection dynamics of *Heligmosomoides polygyrus* and Wood mouse herpes virus in the natural host, *Apodemus sylvaticus*.

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by Kayleigh Gallagher.

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Abstract

This thesis aims to understand how helminth co-infection affects the susceptibility of wild wood mice to wood mouse herpes virus (WMHV) infection and disease progression, and whether the mechanism behind these within-host parasite interactions is via the host immune response. Co-infection with more than one parasite species is the norm rather than the exception in animal and some human populations, importantly infection with one parasite species has the potential to affect how a host deals with another co-infecting parasite. This is particularly important for helminth-viral co-infections, as they initiate mutually exclusive arms of the host immune system and helminths are well known for modulating the host immune response, hence they are important in co-infection interactions. Here I use a wild wood mouse population (*Apodemus sylvaticus*) which is naturally infected with the helminth, *Heligmosomoides polygyrus,* and WMHV to determine the causes and consequences of *H. polygyrus* – WMHV co-infections in their natural host.

First, I investigated if co-infection with *H. polygyrus* can make individuals more susceptible to WMHV infections using samples from wild wood mice which were naturally infected with both WMHV and/or *H. polygyrus*. These results suggest that animals with higher previous *H. polygyrus* burdens (~3 weeks prior) had an increased probability of having a subsequent WMHV infection, but only in wood mice given prior deworming treatments. This is most likely due to the short-lived effects of ivermectin treatment, with previous studies showing that worm burdens quickly return to pre-treatment levels. Overall, these results show potentially counter-intuitive interactions between *H. polygyrus* burden and deworming treatment, suggesting that there may be complex, dynamic interactions between worm infection and reinfection, and host susceptibility to WMHV, which are sensitive to the timescales of infection and treatment.

Since WMHV infections typically begin with a lytic (active) phase of infection, which is then followed by latent and lifelong infection, I next investigated if infection with *H. polygyrus* increases susceptibility to WMHV infection or increases the probability of having a lytic WMHV infection. I used samples taken from wild wood mice, some of which had received

deworming treatments, to carry out RT-qPCR analysis for open reading frame 73 (*ORF*73) which is one of the few viral genes expressed during viral latency as a marker of latent infection, and PCR for open reading frame 50 (*ORF*50) which encodes the viral gene *rta* (replication and transcription activator) and plays a central role in re-activating the virus from latency. This data was used alongside data already collected on the individuals *H. polygyrus* infection status to determine whether *H. polygyrus* co-infection affected the likelihood that WMHV infections were in the lytic or latent phase of infection. I found that effective deworming treatments can reduce the probability of having a WMHV co-infection. However, there was no suggestion that *H. polygyrus* co-infection or deworming treatments had any effect on the probability of having a lytic WMHV infection in this wild wood mouse system.

To further explore the underlying mechanisms of helminth-viral co-infection interactions in this wild wood mouse system, I used RT-qPCR for some Th1 (anti-viral) and Th2 (induced by helminth infection) cytokine genes, to determine if co-infection with *H. polygyrus* results in the downregulation of the anti-viral Th1 arm of the immune system. I found that in this wild wood mouse population, mice with current or previous *H. polygyrus* co-infections had reduced Th1 cytokine gene expression (TNF-α and IL-6) compared to those with only WMHV infections. Additionally, there was no evidence of Th2/immunoregulatory cytokines being upregulated in response to *H. polygyrus* infection, indeed the immunoregulatory cytokine TGF-β was upregulated in mice with WMHV-only infections. Overall, these results suggest that there could be a lag between upregulation of Th2 cytokines and the downregulation of Th1 cytokines which our trapping time scales were not able to capture.

Finally, I used controlled infection/co-infection experiments, with an outbred wild-derived, but now laboratory reared colony of wood mice, to further investigate these within-host interactions under both high- and low-quality nutritional provisioning. Overall, these results show that *Heligmosomoides bakeri* co-infection reduces the ability of the host to control WMHV in mice on lower quality diets, but not in mice on higher quality diets. Additionally, I found that being co-infected with *H. bakeri* was associated with mice on both diets having a longer period of lytic infection in the lungs, however during the early stages of WMHV infection, co-infection seems to enable to host to suppress lytic virus in the lungs to lower levels. Furthermore, there was some evidence of co-infected mice having higher Th2 (IL-5)

expression, however I found the Th1 response to co-infection was very mixed. Overall, *H. bakeri* co-infection had a negative impact on the host ability to deal with WMHV co-infections, and this was particularly apparent in mice on lower quality diets. Despite this there was no apparent reduction in Th1 cytokine responses, suggesting there could be other immune factors at play which contributed to the increased susceptibility to WMHV infection seen this in wood mouse system.

Taken together, these results show how complex within-host co-infection interactions can be and why it is particularly important to study these interactions in the natural host. They also highlight that whilst the host immune response can play a critical role in determining the outcomes of co-infections, other factors such as host nutritional status can also play an important role. This also provides additional support to moving away from artificially clean SPF laboratory studies, and instead using wild or 'dirty' lab animals, whose immune systems are challenged by co-infections and/or poor nutrition, and who are the natural hosts for the parasites. Thus, providing a more natural model of infection, with the hope that these results will more accurately capture what is seen in wild animal and human populations.

1. General Introduction

1.1 Introduction

Co-infection of hosts by multiple parasite species is extremely common in nature (Cox 2001). Many animals and humans harbour infections of both macroparasites such as helminths, and microparasites which include viruses, bacteria, and protozoa. Helminths are frequently found co-infecting individuals infected with microparasites such as malaria, tuberculosis (TB) and HIV (Brown et al. 2006, Hartgers and Yazdanbakhsh 2006, Griffiths et al. 2015). Furthermore, parasitic helminths can have powerful effects on the host's immune system, potentially altering the host's ability to fight microparasitic infections (Spiegel et al. 2003, Ezenwa et al. 2010). Unsurprisingly, this can lead to within-host interactions between the co-infecting parasites, potentially resulting in the host having increased susceptibility to, or morbidity from, microparasitic infections. For instance, infection with one parasite can alter a host's ability to respond to co-infection with another parasite (Salgame et al. 2013, Guivier et al. 2014).

Given this, there is a great scientific and applied interest in understanding, whether, and how, co-infecting macroparasites and microparasites interact simultaneously in each host. Many studies on HIV/AIDS sufferers have found evidence suggesting that immunological responses to helminth infection may predispose an individual to HIV infection (Fincham et al. 2003) or increase HIV viremia levels in an already infected individual (Mulu et al. 2013). Hence, making them more susceptible to infection and/or disease progression. Additionally, Su et al. (2014) found that mice co-infected with the nematode *Heligmosomoides polygyrus* and the bacterium *Salmonella enterica,* serovar *typhimurium* had more severe pathology and a higher mortality rate, compared to mice infected with *S. typhimurium* alone. Similarly, Ezenwa et al. (2010) found that gastrointestinal (GI) nematodes in African Buffalo induced immune suppression which affected the immune response needed for protection against microparasitic infections. This immunosuppression by the nematode resulted in an increased probability of an individual becoming infected with TB; in buffalo without GI nematode infections TB was predicted to fail to establish an infection (Ezenwa et al. 2010).

1.2 Host immunity to macroparasites and microparasite infections, and potential for immune mediated interactions between them.

Helminths are macroparasites that can potentially cause persistent asymptomatic or subclinical chronic infections which may be lifelong (Moreau and Chauvin 2010). Helminths and other macroparasites in general are dealt with differently by the host immune system compared to most microparasitic infections (Figure 1.1). Broadly, microparasites tend to induce a pro-inflammatory T-helper cell type 1 (Th1) immune response which is essential for the control of viral infections, and results in increased levels of the cytokines IL-2, IL-23, tumor necrosis factor (TNF)-α, and interferon (IFN)- γ (Anthony et al. 2007, Alves et al. 2009, Moreau and Chauvin 2010). Some Th1 responses have now been split into a 'new' T-cell subset, called Th17 cells, which are distinct from Th1 cells and are crucial for controlling microparasitic infections (Milner et al. 2008, Dienz et al. 2012); in particular they secrete IL-17 along with pro-inflammatory cytokine TNF-α (Kolls and Lindén 2004, Langrish et al. 2005, Anthony et al. 2007). Recently, it has been found that TGF-β1 is important in activating Th17 cells making it important in the pro-inflammatory response (Eisenstein and Williams 2009). These cytokines produced during a Th1 immune response can activate $CD8^+$ cytotoxic T cells which kill virus-infected cells, induce neutralising IgG antibodies against virus particles, and cause inflammation. In contrast, helminths tend to induce T-helper cell type 2 (Th2) immune responses. In mice infected with *H. polygyrus* group-2 innate lymphoid cells (ILC2s) expand in the intestinal lamina propria (LP) during early infection invoking a local Th2 response (Pelly et al. 2016). Additionally, ILC2s are important for Th2 cell differentiation, they produce significant amounts of IL-4 and IL-2 following *H. polygyrus* infection which is required for optimal Th2 cell differentiation (Pelly et al. 2016). The Th2 immune response is characterised by the proliferation and activation of T cells that secrete Interleukin (IL) 4, IL-5, IL-9 and IL-13 (Anthony et al. 2007). These Th2 cytokines enable helminth expulsion from the gut by inducing eosinophil differentiation, activating B cells (Chan et al. 1992, Anthony et al. 2007), and inducing alternatively activated macrophages (Weng et al. 2007). Additionally, helminth infections are known to induce T-regulatory cells (Treg) to produce TGF-β which has been found to down regulate both Th1 and Th2 immune responses (Su et al. 2005, Anthony et al. 2007). IL-6 also plays an important role in the Th2 response by promoting naïve T cell to differentiate into Th2 cells via IL-4, additionally IL-6 can inhibit Th1

responses by upregulating suppressor of cytokine signalling (SOCS)-1 expression which interferes with INF- γ production and differentiation of Th1 cells (Velazquez-Salinas et al. 2019).

Importantly, there may be interactions between these different components of the immune response (Figure 1.1, red arrows). This means that prior infection with helminths may affect the ability of the host to respond to subsequent microparasitic infections. The Th2 cytokines, as well as immune-regulatory pathways initiated by infection with helminths (such as those that activate T-reg cells), have the potential to downregulate the effector functions (e.g. Th1 cytokines such as IFN- γ) that induce resistance to microparasites during a Th1 immune response (Salgame et al. 2013). Taken together, the downregulation of the Th1 immune responses by Th2 cytokines could impair the ability of the host's immune system to cope with microparasite co-infections such as viruses, thus exacerbating the impact of those viral infections. Thus, these complex immune interactions between helminths and microparasites are thought to be the main drivers of many of the within-host parasite interactions seen in laboratory and wild animal models of co-infection.

Helminths are prime candidates for causing immune-mediated within-host interactions during co-infection with microparasites such as viruses, bacteria or protozoa, due to their well-studied immunomodulatory effect on the host (Maizels et al. 2004, Elliott et al. 2007, Maizels et al. 2012a). Studies using laboratory animals have demonstrated that helminths can exacerbate disease severity caused by viral infections. For example, Actor et al. (1993) infected mice that had previously been infected with *Schistosoma mansoni,* with a recombinant vaccinia virus expressing the HIV-1 protein gp120 and it was found that co-infected mice could take as much as 3 weeks longer to clear the virus, compared to mice only infected with the vaccinia virus. Additionally, when spleen cells taken from mice infected with only vaccinia virus were stimulated with gp120 they produced higher INF- γ and IL-2, but lower IL-4 responses than the mice infected with the *S. mansoni* only. However, those infected with both the vaccinia virus and *S. mansoni* produced lower INF- γ and IL-2 responses when challenged with gp120 than the vaccinia virus alone, but produced no IL-4 response above the non-specific response displayed by *S. mansoni* only infected mice (Actor et al. 1993).

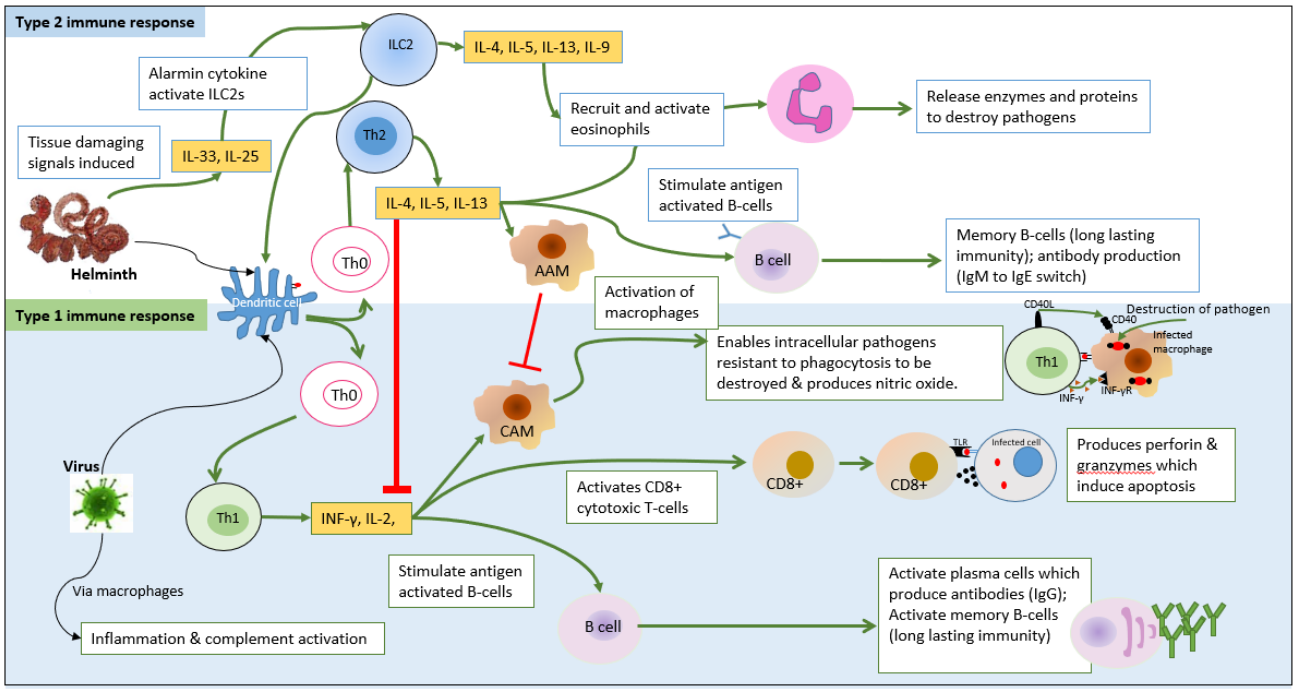

Figure 1.1. Th2 immune response produced in response to helminth infection can downregulate the Th1 immune response aimed at controlling viral infections. Broadly, parasites are presented to cellular components of the immune system via professional antigen presenting cells (APCs), such as dendritic cells in the context of MHC class II molecules to naïve T cells (Hivroz et al. 2012). Naïve T cells then become activated into different T cell subsets depending on the type of pathogen/immunogen. Generally helminths initiate Th2 immune responses including cytokines such as IL-4, 5 and 13 (Anthony et al. 2007). In contrast, viruses promote a Th1 immune responses producing IL-2 and INF- γ (Palm et al. 2012). Moreover, Th2 immune responses can downregulate Th1 responses (Salgame et al. 2013). The cytokines produced during a Th2 immune response enable helminth expulsion from the gut by stimulating antigen activated B cells to produce antibodies and antibody class switching from IgM to IgE (McHeyzer-Williams and McHeyzer-Williams 2005), recruiting and activating eosinophils to destroy pathogens (Gause et al. 2003). The Th2 response also promotes alternatively activated macrophages (AAMs) (McSorley et al. 2013) which are detrimental to helminths (Anthony et al. 2006). The Th1 response also activates $CD8^+$ cytotoxic T cells which will detect virus infected cells using the T cell receptor (TLR) and induce cell death by apoptosis (Nair et al. 2010), stimulate B cells to produce neutralising IgG antibodies (Nair et al. 2010). Additionally, alarmin cytokines induced by exposure to helminths activate ILC2s which secrete Th2 cytokines support the differentiation of naïve T-cells into Th2 cells, recruit and activate eosinophils, and enable dendritic cells to induce Th2 cell differentiation (Gurram et al. 2019). The Th1 responses can also promote classically activated macrophages (CAMs) by engaging the IFN- γ receptor (IFN- γR) and CD40 ligand (CD40L) which in turn can eliminate intracellular pathogens (via nitric oxide (NO)) which are resistant to phagocytosis (Mantovani et al. 2005). Alternatively activated macrophages can also have a negative impact on CAM activation (De'Broski et al. 2004). Red arrows represent inhibitory interactions of the Th-2 immune response on the Th1 response, and hence a potential immune-mediated pathway of interaction between macro- and micro-parasites, facilitating microparasite infection.

Another study using mice co-infected with *S. mansoni* and lymphocytic choriomeningitis virus (LCMV) found that co-infected individuals showed more sever clinical outcomes and the liver was more susceptible to virus replication, with increased liver damage in co-infected mice compared to those with only single infections (Edwards et al. 2005). Another example using Rhesus monkeys with stable simian-human immunodeficiency virus (SHIV) found that animals with clinically stable infections (no sign of immunodeficiency or detectable viral RNA), developed spikes in viral RNA levels when co-infected with *S. mansoni* (Ayash-Rashkovsky et al. 2007). Moreover, Using mRNA expression to determine levels of Th1 or Th2 specific cytokines from blood samples they found that levels of the Th2 cytokine IL-4 were higher in individuals with concurrent *S. mansoni* infections compared to virus only infected (Ayash-Rashkovsky et al. 2007). However, there were no differences in levels of the Th1 cytokines (IL-2 and INF-γ) between singly and co-infected individuals (Ayash-Rashkovsky et al. 2007).

The evidence indicates that infection with a Th2 inducing macroparasite has the potential to downregulate the pro-inflammatory Th1 arm of the host immune response, with consequences for co-infecting microparasites. However, even in these highly controlled laboratory experiments results can vary. For example, Scheer et al. (2014) studied the effect of murine Schistosomiasis on subsequent infection with influenza A virus or pneumonia virus of mice (PVM), they found that mice infected with *S. mansoni* 12 weeks before viral infection survived what would normally be lethal doses of influenza A and PVM. Additionally, the authors found a correlation between *S. mansoni* egg production and TNF-α-mediated goblet cell hyperplasia and mucus secretion, suggesting that these processes may infer protection to viruses of the respiratory system (Scheer et al. 2014). These studies show that infection with a parasite that causes a Th2 immune response has the potential to alter the Th1 immune response needed to control co-infecting viruses, therefore potentially increasing/decreasing viral replication and disease severity in laboratory animals. However, we do not know whether or how these findings in the laboratory relate to wild animals in the field.

1.3 Translating from lab to field

Most of our understanding of the how co-infecting parasites interact come from laboratory models of infection, however laboratory animals have very different immune responses compared to wild animals, thus raising the question of whether these interactions, measured in laboratory mice, are detectable in their natural host populations. Interestingly, laboratory mice raised in SPF conditions have been found to have immune systems more closely related to those of neonatal humans than adult humans (Beura et al. 2016a). This should not be surprising since animals in their natural environment will typically be infected with numerous parasite species throughout their lives. Conversely, laboratory mice have relatively naïve immune systems since they have far less experience with infectious disease than free-living animals would naturally acquire (Babayan et al. 2011, Pedersen and Babayan 2011, Maizels and Nussey 2013, Cadwell 2015, Beura et al. 2016a). Additionally, laboratory mice have limited genetic diversity and receive a constant supply of food and water (Pedersen and Babayan 2011, Maizels and Nussey 2013, Cadwell 2015), whereas wild animals have much more genetic diversity, variable amounts of food and water and diverse environmental conditions (Cox 2001, Ezenwa et al. 2010, Telfer et al. 2010). Hence, to fully understand the host immune response to infections and how it drives co-infection dynamics we may need to move more towards 'dirty' or wild mice for immunological studies.

While most of our understanding of co-infections comes from laboratory studies there are some studies which have used wild animals. For example, Guivier et al. (2014) used wild vole populations and found a positive association between *Heligmosomum mixtum* and Puumala Hantavirus infections (PUUV), whereby those infected with *H. mixtum* where more likely to become infected with PUUV. Similarly, Risco et al. (2014) found that wild boars co-infected with bovine tuberculosis (bTB) and a nematode species (*Metastrongylus* spp.) had an increased infection severity than those only infected with bTB (Risco et al. 2014). Furthermore, there have been studies using human patients with helminth and viral co-infections. Although the results are somewhat more conflicting, with some finding co-infection was associated with increased viral loads (Mulu et al. 2013), and decreased virus specific immune responses (Farid et al. 2005), and others finding lower viral loads

(Modjarrad et al. 2005) or no effect of co-infection on viral disease progression (Hosseinipour et al. 2007).

1.4 Deworming in the context of co-infection interactions

Unsurprisingly, the growing evidence around helminth infections having an impact on co-infecting microparasites has brought into question whether the severity of, and susceptibility to, infection with microparasites could be altered using anti-helminthic drugs (Nacher 2006), and as a result there have been calls for deworming to be incorporated into programmes aimed at controlling major diseases caused by microparasitic infections (Gerns et al. 2012). Helminth infections are common infections of poor people in the developing world and can cause a considerable amount of disease burden in human populations (Brooker 2010). Consequently, there have been calls for mass deworming to be introduced into control measures against major viral, bacterial or protozoal diseases (Molyneux et al. 2005, Hotez 2009). For example, some studies show reduced HIV viremia (Mulu et al. 2013), and increased $CD4^+$ cell count (Blish et al. 2010) in anthelminthic treated individuals. Additionally, Wolday et al. (2002) studied the effect of anthelminthic treatment on HIV viral load in individuals co-infected with helminths and HIV in Ethiopia. They found that six weeks after treatment there was a significant change in HIV viral loads between treated and non-treated individuals; treated individuals had lower viral loads. However, the benefits of these integrated control programmes are debated, mainly due to conflicting results on the outcomes of deworming co-infected individuals since interactions between helminths and microparasites can also be negative (Pedersen and Fenton 2007). Furthermore, Modjarrad et al. (2005) found that there was no association between the treatment of helminth infections and reduced HIV viral load in adults in Zambia.

These studies show how variable the outcomes of deworming can be. Hence, there is a need to better understand how microparasite and macroparasite co-infections affect disease outcomes in each host and what targeted therapeutic interventions will provide the best protection/resolution in such cases. Studies in wild animal populations can serve as a suitable and tractable model to explore, not only how these co-infections in a given host may affect the overall immunological fitness by assessing helminth-microparasite

interactions- but also determine the effects of different treatment strategies that are envisaged will be relevant to co-infections in human populations.

1.5 Wood mice as a suitable model system to study helminth-microparasite co-infection dynamics.

The wood mouse (*Apodemus sylvaticus*) is a common free-roaming wild mouse species in the UK and has proved invaluable to both ecological and immunological studies. In the UK, most wood mice naturally harbour both helminth and viral co-infections and they can be readily treated using deworming drugs (Knowles et al. 2013). The anthelmintic drugs ivermectin (Wahid and Behnke 1993) and pyrantel (Wahid et al. 1989) are widely used to clear *H. polygyrus* infections. Ivermectin targets the larval stages of infection, whereas pyrantel targets adult worms (Wahid et al. 1989). This makes wood mice a suitable model to assess the interactions between helminths and viral-pathogens, and explore the effects of mass deworming on helminth-viral dynamics (Su et al. 2006, Knowles et al. 2012). Many wood mouse populations in the UK are infected with Wood Mouse Herpes Virus (WMHV) and the helminth *Heligmosomoides polygyrus* (Knowles et al. 2012). Given the ubiquity of infection, and co-infection between these parasites, these is considerable potential for within-host interactions between them.

WMHV is a gamma-herpesvirus (γ-HV), belonging to the family *Herpesviridae* and is most commonly found in wood mice and other murid rodents (Telfer et al. 2007). There are over 70 species of virus in the Herpesvirus family, and eight of these are pathogenic to humans, with 30%-90% of the adult population being infected with at least one herpesvirus (Shukla and Spear 2001). Importantly for this project, murine herpesvirus 60 (MHV-68 or the laboratory strain of WMHV) has been found to be closely related to Human Herpes virus-8 (HHV-8; also known as Kasposi's sarcoma-associated herpes virus or KSHV), Epstein-Barr virus (EBV) and non-human Saimiriine herpes virus-2 or Herpes Virus Saimiri (HVS-2) (Efstathiou et al. 1990, Virgin et al. 1997, Nash et al. 2001). Given the ease with which MHV-68/WMHV can be cultured in tissue culture grown cells, it is an ideal model to study these closely related viral pathogens.

Although the natural route of WMHV transmission is not entirely clear, WMHV viral antigens have been readily detected in upper respiratory tract and lung alveolar epithelial cells in

free roaming wood mice. Therefore, a preferred route of infection of laboratory mice with MHV-68 has been the respiratory route (Nash et al. 2001). Gamma-herpes virus infection in mice typically begins with a lytic phase which involves productive viral replication in the lungs (Sunil-Chandra et al. 1992a). Lytic virus replication in the lungs is usually cleared by 10 days post infection (Sunil-Chandra et al. 1992a, Usherwood et al. 1996a). From the lungs, the virus then spreads to the spleen were a latent and lifelong infection is established, mainly in splenic B cells, but also in macrophages and dendritic cells (Flaño et al. 2000, Nash et al. 2001, Flaño et al. 2005). During viral latency, very few viral genes are expressed therefore allowing it to evade immune control, but also reducing the likelihood of virus transmission to other individuals (Nash et al. 2001). However, the latent virus has the molecular ability to reactivate into the lytic infection, so re-enabling it to infect new hosts (Wu et al. 2000). While very few viral genes are expressed during latent infection, open reading frame 73 (*ORF*73) is critical to establish and maintain viral infection in latently infected cells (Fowler et al. 2003, Marques et al. 2003). Additionally, the viral gene *rta* (replication and transcription activator) plays a central role in re-activating the virus from latency and is primarily encoded by open reading frame 50 (*ORF*50) (Liu et al. 2000, Wu et al. 2000, Wu et al. 2001). Given the similarities to pathogenic human gamma-herpesviruses and the ease with which MHV-68 can be maintained and used for controlled infection experiments, MHV-68 has become widely used as a suitable model to understand other gamma-herpesviruses and their host-parasite interactions.

MHV-68 infection in laboratory mice usually starts with a lytic phase of infection (most viral genes are expressed, and the virus can replicate) in the lungs before moving to the spleen (Sunil-Chandra et al. 1992a, Flaño et al. 2005) (Figure 1.2). Lytic virus replication in the lungs can be detected from 1 day post infection (dpi), with peak titres at 7 dpi and is usually cleared from the lungs by 10 dpi (Flaño et al. 2005). Furthermore, latent (non-replicating) virus has been found in the lungs of mice from as early as 3 dpi with levels of latent virus remaining high before declining to low stable levels by 30 dpi (Flaño et al. 2005). From the lungs the virus then spreads to the spleen, with infectious virus detectable at low levels from 3 dpi, with latent virus detectable from 6 dpi and peak latent infection is reached around 14 dpi before declining to low stable levels by day 90 days post infection (Sunil-Chandra et al. 1992a, Flaño et al. 2005). WMHV then establishes a latent and lifelong

infection, mainly in splenic B cells, but also in macrophages and dendritic cells (Flaño et al. 2000, Nash et al. 2001, Flaño et al. 2005) and lung epithelial cells (Stewart et al. 1998). The lifecycle of rodent gammaherpes viruses was investigated in laboratory mice, however Hughes et al. (2010b) studied the progression of lytic MHV-68 infection in the lungs and latent infection in the spleens of wood mice, and found that progression of MHV-68 in wood mice is similar to that in laboratory mice. Noticeably, the main differences were that infectious virus in the lungs could not be detected until day 7 and there were lower levels of latent virus in the spleen after 20 days post infection (Hughes et al. 2010b).

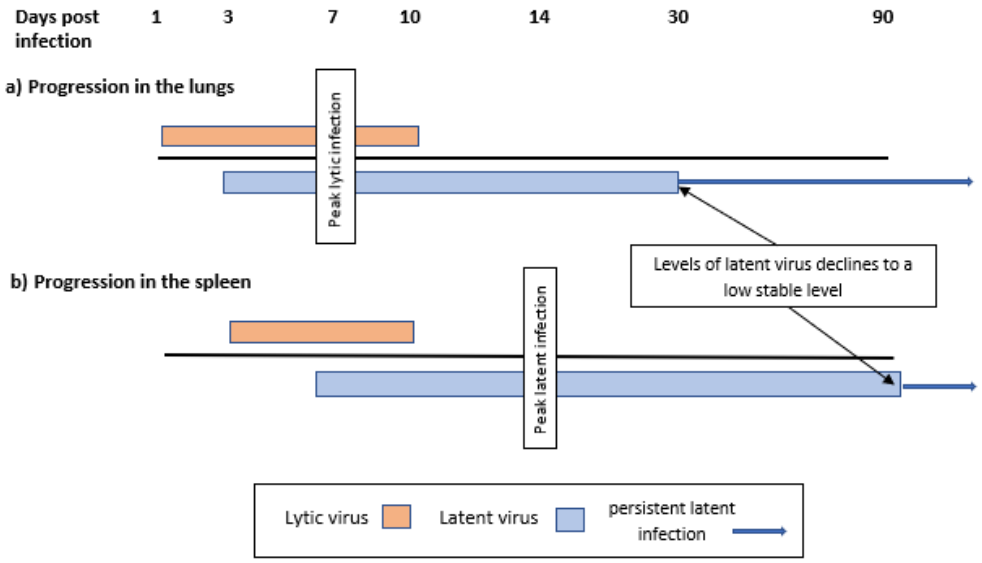

Figure 1.2. MHV-68 infection progression in the spleen and lungs of mice. **A) Progression in the lungs:** Lytic virus can be detected from 1 day post infection (dpi) with peak titres at 7 dpi and infection cleared from the lungs by 10 dpi, and latent virus can be detected from 3 dpi with levels remaining high through the lytic phase and declining to low stable levels from 30 dpi (Flaño et al. 2005). **B) Progression in the spleen:** Infectious virus is detectable at low levels from 3 dpi with latent virus detectable from 6 dpi, and peak latent infection is reached around 14 dpi before declining to low stable levels by 90 dpi (Sunil-Chandra et al. 1992a, Flaño et al. 2005).

Nematodes of the genus *Heligmosomoides* are very adept at modulating the host immune system (Monroy and Enriquez 1992). A member of this genus, *H. polygyrus* is a murine intestinal nematode that naturally occurs in wild mouse populations in the UK and across Europe (Maizels et al. 2012b), and typically at relatively high prevalence; approximately half of wild wood mice in the UK carry *H. polygyrus* infections (Knowles et al. 2013). The lifecycle of *H. polygyrus* begins when a mouse ingests infective larvae (L3) which pass through the digestive tract to the small intestine (Reynolds et al. 2012). The larvae penetrate though the wall of the small intestine to the submucosa, where they undergo two molts and by day 10

post ingestion they return to the gut lumen as adult worms, they then mate and the female worms release eggs (Reynolds et al. 2012). These eggs are released into the environment in the hosts faeces, the larvae then emerge from the eggs and two molts are needed before the larvae are at the infective L3 stage and ready to infect a new host (Reynolds et al. 2012).

Many laboratory studies have been conducted using *H. bakeri* (formally *H. polygyrus bakeri*) which is a closely-related laboratory species of the wild species, *H. polygyrus* found in European wood mice (Behnke and Harris 2010, Maizels et al. 2012b). *H. bakeri* is used in many laboratory models because most laboratory bred mice are unable to expel primary infections, thus providing a stable host-parasite system in which to analyse the immune mechanisms of chronic helminth infection (Maizels et al. 2012b). Additionally, *H. bakeri* is well known for its immunosuppressive abilities in laboratory mice, because it can modify a wide range of host immune responses (Finney et al. 2007, Segura et al. 2007, Grainger et al. 2010). Therefore, I aim to use this model system to study the effects of helminth co-infections on susceptibility to viral infections and disease progression.

1.6 Co-infection outcomes can affect parasite and host population dynamics.

As co-infection has the potential to change within-host infection dynamics, this could potentially have consequences for between host dynamics and epidemiological dynamics. For example, if macroparasite and microparasite co-infection results in more infectious particles (microparasite) being released into the environment then co-infection could result increased microparasite transmissibility. Fenton (2008) found that in models where the presence of a macroparasite favours microparasite transmission, then the microparasite was predicted to be able to persist, even when it had no effective transmission without the macroparasite co-infection. It was also predicted that if co-infection increases host mortality then there was a reduced chance of microparasite persistence (Fenton 2008). Additionally, within-host interactions has noticeable consequences for the abundance of both host and parasite species, this could potentially result in a reduction of the host population abundance (Fenton 2008). These results suggest that if there are co-infections interactions in our wood mouse system then there could be consequences for parasite and host population dynamics. For example, Reese et al. (2014) found that H. bakeri co-infection re-activated MHV-68 from latency, this could have consequences for microparasite

transmission rates since MHV-68 is only transmissible to others when in the lytic stage of infection.

1.7 Aims and objectives of research project

Clearly, co-infection can have an impact on the host's immune system and eventual disease outcome. Helminth infection potentially increases susceptibility to infection, disease progression, and parasitaemia levels for viruses, bacteria, and protozoa. A suitable system assessing helminth-viral interactions in the natural setting, as well as determining the effects of different treatment scenarios is needed, and wild rodent models are ideal for this. A vast amount of background information and logistics have previously been worked out using wild rodent models and in particular wild wood mice, hence this allows helminth-virus interactions to be assessed and alternative treatment scenarios explored.

This study aims to test the overarching hypothesis that natural *H. polygyrus* infections reduce a host's ability to deal with co-infecting WMHV via the host immune system. This will be done by addressing the following specific objectives:

1) Determine if helminth infection increases the likelihood of becoming infected with WMHV in the field, using immunofluorescent assays on a substantive set of longitudinally collected wood mouse sera samples collected over the period 2009-2014.
2) Determine if helminth co-infection and/or deworming influences the likelihood of being infected with WMHV, or WMHV-infected individuals having a lytic WMHV infection, by carrying out RT-qPCR and PCR analysis on wild wood mouse spleens in order to detect expression of viral *ORF50* and *ORF73* genes.
3) Determine the mechanism of interaction between *H. polygyrus* infection and WMHV susceptibility, using RT-qPCR to detect expression of key cytokines produced by a Th1 and Th2 immune response. These data were used to determine if co-infected mice have reduced Th1 and/or elevated Th2 responses compared to those infected only with WMHV.
4) Determine the effect of chronic and cleared (using ivermectin treatment) *H. polygyrus* infections on WMHV infection dynamics, using laboratory raised wood

mice experimentally infected with WMHV and *H. polygyrus,* additionally some mice were give a standard diet while others were on a high quality diet. Next, we used RT-qPCR to understand how expression levels of key Th1 and Th2 cytokines are affected by *H. polygyrus* co-infection and diet.

2. The effects of *H. polygyrus* infection on susceptibility to WMHV infection in a wild wood mouse population

2.1 Abstract

Co-infection with more than one parasite species is the norm rather than the exception in animal populations, including human populations, particularly those in developing countries. Wild animal populations can be excellent model systems for exploring whether co-infection drives susceptibility and/or infectiousness of major diseases, since they are commonly co-infected by several pathogen species. Parasitic nematodes are often considered to have strong immunomodulatory effects on the host, potentially altering host susceptibility to other, co-infecting parasite species. Here, using samples from wild wood mice which were naturally infected with both wood mouse herpes virus (WMHV) and/or the nematode *Heligmosomoides polygyrus*, I investigated if co-infection with *H. polygyrus* can make individuals more susceptible to WMHV infections. These results suggest that animals with higher previous *H. polygyrus* burdens (~3 weeks prior) was associated with increased probability of having a subsequent WMHV infection, but only in wood mice given prior deworming treatments. This is most likely due to the short-lived effects of ivermectin treatment, with worm burdens quickly returning to pre-treatment levels. There was though no evidence of a direct effect between *H. polygyrus* infection and the likelihood of becoming infected with WMHV. Overall these results show potentially counter-intuitive interactions between *H. polygyrus* burden and deworming treatment, suggesting that there may be complex, dynamic interactions between worm infection and reinfection, and host susceptibility to WMHV, which are sensitive to the timescales of infection and treatment.

2.2 Introduction

Historically, disease dynamics were typically studied using the classical one-host-one parasite approach (Pedersen and Fenton 2007). However, co-infection with more than one parasite species is the norm rather than the exception in animal populations (Petney and Andrews 1998), including human populations, particularly those in developing countries (Mupfasoni et al. 2009, Griffiths et al. 2011). Co-infection allows for potential within-host interactions between parasite species, which can affect disease progression (Modjarrad et al. 2005), and disease severity (Su et al. 2014). Additionally, co-infection can shape disease epidemiology by altering parasite dynamics, and their rate of replication and transmission (Altizer and Pedersen 2008). These interactions can either be direct, such as competition for space between species that occupy the same physical location within the host, or indirect via resource competition or the immune system (Pedersen and Fenton 2007). Furthermore, although some individuals may not be simultaneously co-infected with two parasite species, prior infection history may play a role in determining the outcome of current infection, for example due to the longer-term impact of changes to the host's immune system (Walzl et al. 2000). Since co-infections can have a considerable impact on the clinical outcomes of disease, recent 'One-Health' frameworks highlight the importance of multi-parasite systems, and suggest they be accounted for in all human and animal disease control plans (Webster et al. 2016). In particular, having a better understanding of how co-infecting parasites interact, and how they affect key parameters such as parasite transmission and disease progression will allow for better model predictions and more efficient treatment programmes (Lello and Hussell 2008).

2.2.1. *Helminth-microparasite interactions*

Helminths in particular are prime candidates for causing immune-mediated within-host interactions during co-infection with microparasites such as viruses, bacteria or protozoa, due to their often-strong immunomodulatory effect on the host (Maizels et al. 2004, Elliott et al. 2007, Maizels et al. 2012a). Several laboratory studies using helminth and viral species have demonstrated that, in terms of host susceptibility to infection and disease progression, helminths can have a negative impact on the outcome of viral infections. For example, mice

co-infected with *Schistosoma mansoni* and lymphocytic choriomeningitis virus (LCMV) showed more severe clinical outcomes, increased susceptibility to virus replication, and increased liver damage than mice with only single infections (Edwards et al., 2005). Additionally, co-infection with helminths has been shown to increase viral RNA levels (Ayash-Rashkovsky et al. 2007), increase time taken for hosts to clear virus infections (Actor et al. 1993), more serve viral pathology and increased mortality (Edwards et al. 2005), and decreased production of the cytokines interferon (INF)- γ and Interleukin (IL) -2 resulting in delayed virus clearance (Actor et al. 1993). These studies all show a negative impact of helminth co-infection on the outcome of viral infections for the host, however there are some studies that show conflicting results. For example, Scheer et al. (2014) studied the effect of murine schistosomiasis on subsequent infection with influenza A virus or pneumonia virus of mice (PVM). They found that mice infected with *S. mansoni* 12 weeks before viral infection survived what would normally be lethal doses of influenza A and PVM. The mechanism of these interactions is thought to be via the host immune system (Salgame et al. 2013). Helminths are dealt with differently by the host immune system than microparasites. Helminths tend to stimulate a type 2 (Th2) immune response which produces messenger molecules (cytokines) which play a role in activating immune effector functions aimed at controlling helminth infections (Ezenwa et al. 2010). Whereas, microparasites tend to produce a type 1 (Th1) immune response, which produces cytokines aimed at controlling microparasitic infections (Ezenwa et al. 2010). These arms of the immune system can be mutually inhibitory, meaning the helminth induced Th2 responses can potentially increase susceptibility to microparasites by downregulating the Th1 immune response (Spiegel et al. 2003, Ezenwa et al. 2010). Consequently, there is a great scientific, and applied, interest in understanding, whether, and how, co-infecting macroparasites and microparasites interact in each host.

2.2.2. *Wood mice as a suitable model system for studying helminth-viral interactions in the wild*

Wild animal populations can be excellent model systems for exploring whether co-infection drives susceptibility to, and infectiousness of, major diseases; since they are commonly co-infected by several pathogen species (Bordes and Morand 2011). The wood mouse (*Apodemus sylvaticus*) is a common free-roaming wild mouse species in the UK and has

proved invaluable to both ecological and immunological studies. Most wood mice in the UK are naturally co-infected with both helminths and viruses, and they can be readily treated using deworming drugs to explore the effects of helminth removal on susceptibility to other parasite species (Knowles et al. 2013). Additionally, wood mice are commonly infected with wood mouse herpes virus (WMHV) and/or the nematode *Heligmosomoides polygyrus*. Both these species have closely related laboratory species that are often used as standard laboratory models of infection, both of herpes viruses and the immunomodulatory effects of helminth infections. Wood mouse herpes virus is a gamma-herpesvirus that is commonly found in mice and other murid rodents (Telfer et al. 2007), and its closely related laboratory species, MHV-68, has been well studied in laboratory mice (Sunil-Chandra et al. 1992b, Reese et al. 2014). WMHV, like most herpesviruses, are able to produce persistent latent infections in the spleen (Sunil-Chandra et al. 1992b), this latent virus then has the molecular ability to reactivate into an active infection (Wu et al. 2000, Wu et al. 2001). Moreover, *H. polygyrus* is known to have suppressive effects on the host immune system (Maizels et al. 2012a), allowing for potential immune-modulated interactions between WMHV and *H. polygyrus*. Taken together, this makes wild wood mice a suitable model to assess the interactions between helminths and viral pathogens in a naturally infected wild animal population (Su et al. 2006, Knowles et al. 2012). Here, I use samples from wild wood mice which are naturally infected with both WMHV and/or *H. polygyrus*, to determine if co-infection with *H. polygyrus* is associated with increased susceptibility to WMHV infections.

2.3 Methods

2.3.1. *Source of wild rodent samples*

This study examined for evidence of WMHV and helminth infections from previously collected blood and faecal samples respectively, from wild mouse sampling conducted every 3 weeks between May and December in 2009-2012 from three mixed woodlands in the UK (Manor Wood, Haddon Wood, and Rode Wood). Woodlands were split into grids, and each grid had two traps (H.B. Sherman 2 x 2.5 x 6.5-inch folding trap) placed every 10m in a 70 x 70m square, and each trap contained grain and bedding. All trapped individuals were tagged using subcutaneous passive integrated transponder tags so they could be identified on

recapture. For every animal trapped a small blood sample was taken from the tip of the tail and a faecal sample collected at each capture. Morphometric details were also taken from each animal then placed in one of the following groups: adult (over 16g), sub-adult (12-16g), or juvenile (under 12g), the sex of each individual, and reproductive state was also recorded. Reproductive status was determined by assessing body shape and by visual examination of genitals. Fecal eggs counts (FEC) were obtained prior to this project using the fecal flotation procedure. Fecal samples are first weighed and formalin added, before adding sample to a fecalyzer and adding a salt solution to separate oocysts from the fecal sample. Next, oocycts are removed from the solution using a cover slip and examined under a microscope for the presence *H. polygyrus* eggs. On some of grids randomly selected individuals were treated with the anthelminthic drug Ivermectin to suppress their helminth infections, with the remaining animals receiving water as a control. All blood samples and demographic data had already been collected prior to the start of this current project.

2.3.2. *Immunofluorescent assay to detect antibodies to WMHV*

Immunofluorescent (IFA) assays were carried out to detect antibodies to WMHV from wild mouse sera samples. IFA assays had been carried out on samples from 2009, 2010 (Knowles et al. 2012), and 2012 (Gallagher 2015) prior to this project; the samples collected in 2011 were analysed as part of this project. The original IFA assay protocol had issues with reproducibility therefore steps were taken to optimise the assay. Firstly, experiments were carried out to determine the number of cells needed to create a 70% confluent monolayer after 24 hours and different plastics were also trailed to determine which the cell monolayer had the best adhesion to. To make the assay more reliable and to reduce any non-specific binding we added in steps to lyses cells using Triton x-100 and then added bovine serum albumin to act as a blocker against unspecific binding. Assay validation was carried out using samples which had previously tested positive for WMHV antibodies, and PBS as a control. The assay first requires NIH-3T3 cells, which are cultured in Dulbecco's Modified Eagle's medium (D-MEM) supplemented with 10µl/ml penicillin streptomycin (Gibco), 20µl/ml L-glutamine (Gibco), 10% heat inactivated foetal bovine serum (Gibco). Monolayers of these cells were grown in 96-well plates, inoculated with 5 p.f.u of MHV-68 virus, and fixed with paraformaldehyde after a 24-hour incubation period before immediately starting the assay.

IFAs were carried out by rehydration of the cells in PBS, then adding 0.3% Triton x-100 to lyse cells before adding 5% (w/v) bovine serum albumin (BSA)/PBS to act as a blocker. Serial dilutions with serum (diluted 1:20 and 1:40) were made, and bound antibodies detected using conjugate anti-mouse/anti-rat IgG FITC (Sigma) diluted in 1% BSA/PBS, before separating by washing in PBS in preparation for viewing under UV illumination. Positive samples were illuminated under UV light (Gallagher 2015).

2.3.3. *Statistical analysis*

All statistical analyses were performed in R, v. 3.6.1 (R development core team 2011).

My primary aim was to understand the relationship between current or prior helminth infections and current or subsequent WMHV infections. Since WMHV infections are lifelong (Nash et al. 2001) we can assume that if animals were seropositive for WMHV antibodies then they were infected with WMHV. Furthermore, since mice were trapped every 2-3 weeks, I could examine how helminth infection or treatment at the previous capture affected WMHV treatment at the subsequent session (2-3 weeks later). I conducted 2 analyses, each examining different aspects of how current and previous *H. polygyrus* infection or FEC affected the probability of being infected, or becoming infected, with WMHV: 1) is current and/or previous *H. polygyrus* infection associated with the probability of also being infected with WMHV (regardless of when the infection occurred)? 2) Does current and/or previous *H. polygyrus* infection affect the probability of an uninfected animal becoming infected with WMHV (i.e., are animals that are WMHV seronegative at one capture more likely to become positive by the next successive capture if they also have *H. polygyrus*)? Current and prior *H. polygyrus* FEC included all individuals regardless of whether they had a *H. polygyrus* infection or not (i.e., zero counts were included in the FEC metric for this predictor variable). Here is a description of each analysis in more detail:

1) Is current and/or previous *H. polygyrus* infection associated with being infected with WMHV?

First, I analysed whether *H. polygyrus* infection or FEC was associated with the likelihood of being infected with WMHV. This ignores when the animal became infected with either parasite and only looks at whether the two infections significantly co-occur in individual mice. This is the coarsest level of analysis considered here but maximises the amount of

data that could be used because it does not require animals to initially be seronegative for WMHV at the first capture, as the other analysis does (see below). Two separate binomial GLM's were created both with WMHV seropositivity at the current session (factor: yes or no) as the response variable, and as the main predictors of interest either current and previous *H. polygyrus* (Hp) infection (absence/presence):

WMHV seropositive ~ Current Hp infection + Previous Hp infection * prior ivermectin treatment + age + reproductive status * weight + year * Session + woodland,

or current and previous *H. polygyrus* FEC:

WMHV seropositive ~ Current Hp FEC + Previous Hp FEC * prior ivermectin treatment + age + reproductive status * weight + year * Session + woodland.

In each case I controlled for age (factor: adult or not), reproductive status/sex (factor: Descended testes (Male), abdominal testes (Male), scrotal testes (Male), pregnant or perforated (female), not pregnant or perforated (female)), weight (continuous), year (4-level factor: 2009-2012), session (factor: June-December), woodland (3-level factor: Manor, Haddon, Rode) and ivermectin treatment (factor: treated or not). Additionally, interactions between these variables were considered and included previous *H. polygyrus* infection*prior ivermectin treatment to determine if the effect of treatment varies depending on previous *H. polygyrus* infection, a reproductive status * weight interaction to check for weight specific reproductive status effects (Knowles et al. (2012) found that heavy males were more likely to be infected with WMHV), and finally a session*year interaction to determine any within year seasonal effects. Ivermectin treatment was not associated with either *H. polygyrus* infection (presence/absence) or FEC, due to the transient impact of treatment on these nematodes (Knowles et al. 2013), so could be included in the models. Full models were simplified by backwards elimination of non-significant terms (initially defined as $p<0.07$), starting with interactions, until a minimal model was obtained with all terms $p<0.05$.

2) Does current and/or previous *H. polygyrus* infection affect the probability of becoming infected with WMHV?

Next, I analysed whether *H. polygyrus* infection or FEC was associated with the likelihood of WMHV-negative animals subsequently becoming infected with WMHV. Only individuals that were seronegative for WMHV antibodies at the previous capture session (2-3 weeks previously) were included in the analysis, allowing me to determine individuals that became infected between successive captures. Using a binomial GLM with WMHV seropositivity at the current session (factor: yes or no), as the response variable separate models were created to first determine the effect of current and previous *H. polygyrus* infection (absence/presence):

*WMHV seropositive ~ Current Hp infection + Previous Hp infection * prior ivermectin treatment + age + reproductive status * weight + year * Session + woodland,*

and second current and previous *H. polygyrus* FEC:

WMHV seropositive ~ Current Hp FEC *+ Previous Hp* FEC ** prior ivermectin treatment + reproductive status * weight + year * Session + woodland,*

on the likelihood of an individual becoming WMHV seropositive (i.e. changing from seronegative at the previous session and to seropositive at the current session). Again, I controlled for the same covariates and interactions listed above, excluding age from the *H. polygyrus* FEC models (since all animals were adults), and simplified the maximal model by backwards elimination to obtain a minimal model with all terms $p<0.05$.

2.4 Results

A total of 2,695 samples were screened using IFA from individual mice that had both blood and faecal samples taken, 607 of these samples were from 2012, 668 from 2011, 485 from 2010, and 935 from 2009 (2009 and 2010 data from Knowles et al. (2012), 2012 data from Gallagher 2015). In order to carry out the full longitudinal analysis described above (analysis 1), data from two successive trapping sessions was needed for each individual to determine how helminth infection at one session affects the likelihood of that individual being WMHV seropositive at the next successive capture, which resulted in sample sizes being reduced (n=1065). Across all years there was a 20.8% WMHV seroprevalence in wood mice and

39.7% had *H. polygyrus* infections. Additionally, between years 2012 had the highest proportion of co-infected individuals while 2010 had the highest number of WMHV only infections (Figure 2.1).

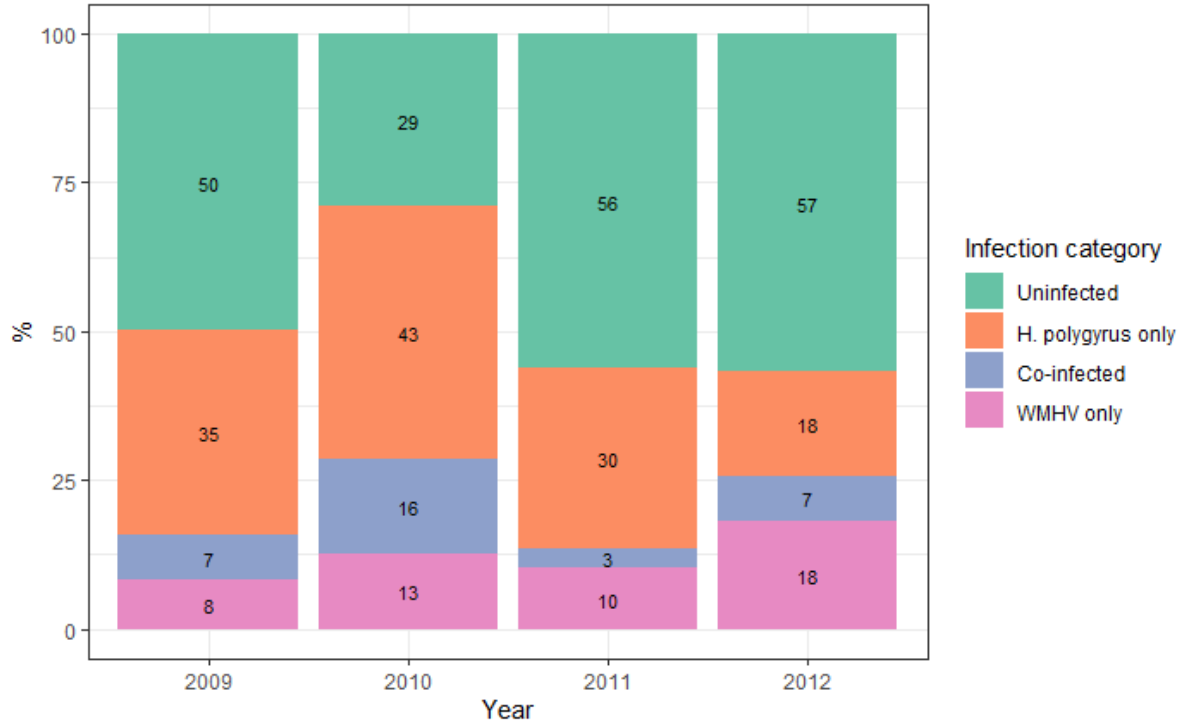

Figure 2.1. Prevalence of un-infected, WMHV only, *H. polygyrus* only and co-infected wild wood mice by year. The numbers in each block represent the percentage of samples in that category (*n*=1065).

2.4.1 *Is current and/or previous H. polygyrus infection associated with being infected with WMHV.*

Overall, no co-infection or treatment factors were found to be associated with WMHV infection. I found no significant association between current or previous *H. polygyrus* infection (current session: x^2_1=0.07, p=0.797; previous session: x^2_1=0.507, p=0.48), or prior ivermectin treatment (x^2_1=0.67, p=0.41) and the likelihood of an individual being infected with WMHV at the current session (Table2a). The only terms that remained in the model were year (x^2_3= 31.76, p<0.001) and a reproductive*weight interaction (x^2_4= 13.63, p= 0.01), which showed that heavier scrotal as males were more likely to have a WMHV infection (Figure 2.2).

In the model containing *H. polygyrus* FEC there was no association between current *H. polygyrus* FEC (x^2_1=0.54, p=0.46) and the likelihood of an individual being infected with WMHV at the current session (Table 2.2b). However, there was a significant previous *H. polygyrus* FEC* prior ivermectin interaction (x^2_1=5.55, p=0.02), with higher previous *H. polygyrus* FEC being associated with increased probability of WMHV infection in treated mice (Figure 2.3). Other variables and interactions that also remained in the model were session (x^2_6= 13.72, p=0.03), year (x^2_3= 30.89, p<0.001), age (x^2_1= 3.96, p=0.05) and weight * reproductive/sex (x^2_4= 13.40, p= 0.01) (Table 2b).

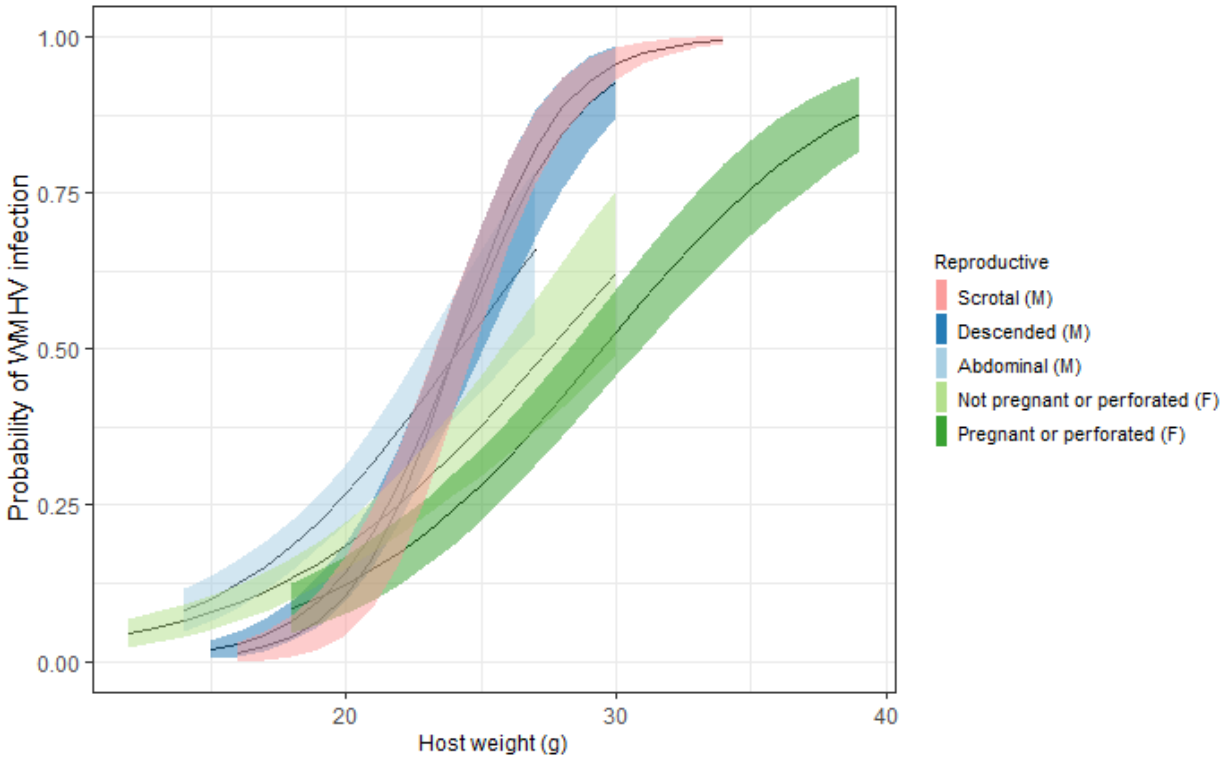

Figure 2.2. Probability of wild wood mice being seropositive for WMHV depending on weight (g) and reproductive status/sex for 2012 (*n*=1065).

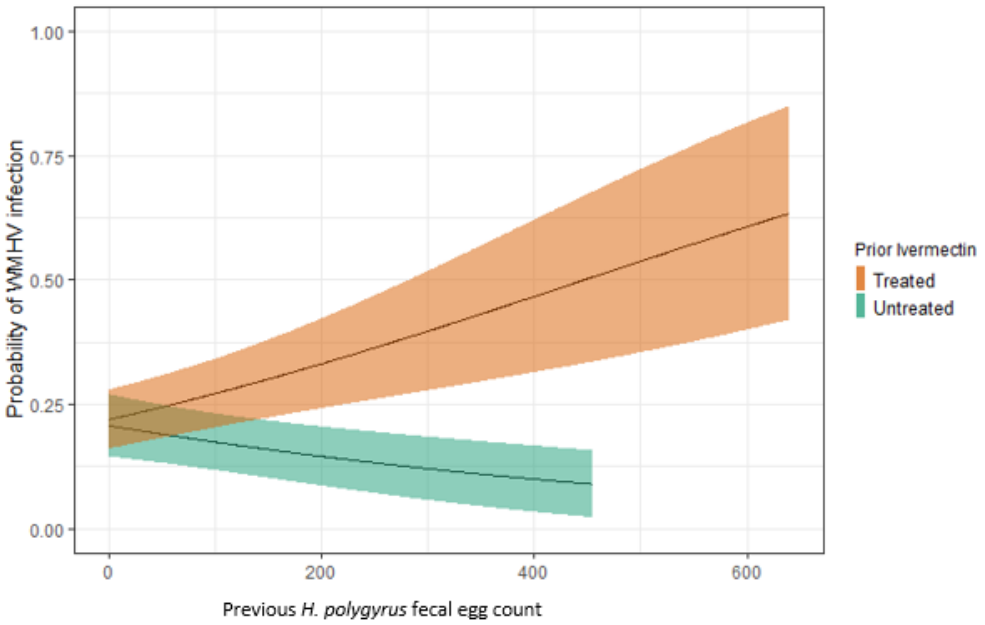

Figure 2.3. Probability of wild wood mice being seropositive for WMHV depending on previous *H. polygyrus* FEC and prior ivermectin treatment for abdominal adult males of mean weight from October 2010 (*n*=485).

Table 2.2. Factors affecting the probability of infection with WMHV in a wild wood mouse population. Models were simplified by backwards stepwise elimination. For variables not in the minimal model, statistics are those at the point they left the model.

a) Model containing *H. polygyrus* presence/absence	df	x^2	P
Weight	1	-	-
Current *H. polygyrus* infection	1	0.0690	0.7929
Previous ivermectin treatment	1	0.6720	0.4124
Previous *H. polygyrus* infection	1	0.5040	0.4780
Woodland	2	4.6040	0.1000
Age (adult or not)	1	3.5490	0.0596
Sex/reproductive	4	-	-
Session * Year	18	14.247	0.7129
Weight (g) * Sex/reproductive ¥	4	13.627	0.0086
Year ¥	3	31.763	<0.001
Session	6	10.968	0.0893
Previous *H. polygyrus* infection * Previous ivermectin treatment	1	1.0440	0.3069
b) Model containing *H. polygyrus* FEC			
Weight	1	-	-
Current *H. polygyrus* FEC	1	0.3063	0.5800
Previous ivermectin treatment	1	-	-
Previous *H. polygyrus* FEC	1	-	-
Woodland	2	4.709	0.0950
Age (adult or not)	1	3.961	0.0466
Sex/reproductive	4	-	-
Session * Year	18	13.768	0.7441
Weight (g) * Sex/reproductive ¥	4	13.400	0.0095
Year ¥	3	30.887	<0.001
Session	6	13.722	0.0329
Previous *H. polygyrus* FEC * Previous ivermectin treatment	1	5.5466	0.0185
¥ Variables in the minimal model (P < 0.05)			

2.4.2 Does current and/or previous H. polygyrus infection affect the probability of becoming infected with WMHV?

Since this analysis only included individuals that were seronegative for WMHV at their previous capture (in addition to being captured at 2 successive sessions as in analysis 1), the sample size was reduced (n=442), with 44 animals becoming positive for WMHV at the next capture (current) session. There was no significant effect of current or previous *H. polygyrus* infection (current session: x^2_1=1.01, p=0.32; previous session: x^2_1=0.13, p=0.72), or prior ivermectin treatment (x^2_1=0.91, p=0.34) on the likelihood of an individual becoming WMHV seropositive at the current session (Table 2.3a). The only term remaining in the model was year (x^2_3=14.31, p<0.01) (Figure 2.4), although these analyses potentially suffer from low power due to small sample sizes.

Additionally, current or previous *H. polygyrus* FEC (current session: x^2_1=0.30, p=0.58; previous session: x^2_1=0.01, p=0.94), or prior ivermectin treatment (x^2_1=0.44, p=0.51) had no effect on the likelihood of an individual becoming WMHV seropositive at the current session (Table 3b). As above the only term remaining the model is year (x^2_3=14.31, p<0.01).

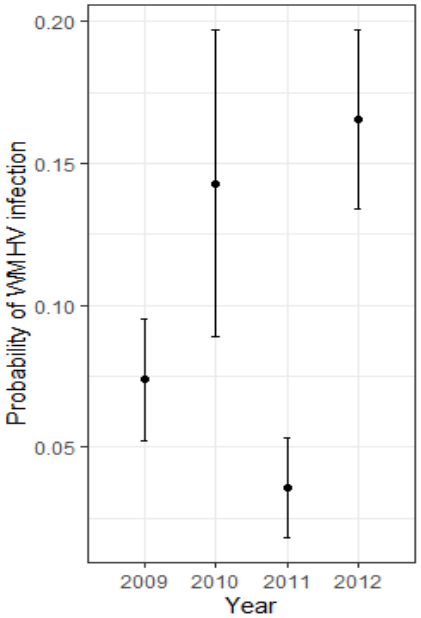

Figure 2.4. Probability of wild wood mice becoming infected with WMHV by year (*n*=1065).

Table 2.3. Factors affecting the probability of wild wood mice becoming infected with WMHV between concurrent trapping sessions (2-3 weeks apart). Models were simplified by backwards stepwise elimination. For variables not in the minimal model, statistics are those at the point they left the model.

a) Model containing *H. polygyrus* presence/absence	df	x^2	P
Weight	1	0.2551	0.6135
Current *H. polygyrus* infection	1	1.0098	0.3150
Previous ivermectin treatment	1	0.9118	0.3396
Previous *H. polygyrus* infection	1	0.1298	0.7186
Woodland	2	2.2722	0.3211
Age (adult or not)	1	0.7370	0.3906
Sex/reproductive	4	4.8036	0.3080
Session * Year	18	8.1223	0.9188
Weight (g) * Sex/reproductive	4	5.3770	0.2508
Year ¥	3	14.312	0.0025
Session	6	6.4563	0.3741
Previous *H. polygyrus* infection* Previous ivermectin treatment	1	0.3425	0.5584
b) Model containing *H. polygyrus* FEC			
Weight	1	0.4316	0.5112
Current *H. polygyrus* FEC	1	0.2985	0.5848
Previous ivermectin treatment	1	0.4351	0.5095
Previous *H. polygyrus* FEC	1	0.0054	0.9413
Woodland	2	2.2722	0.3211
Sex/reproductive	4	4.7189	0.3174
Session * Year	18	8.1892	0.9160
Weight (g) * Sex/reproductive	4	5.0958	0.2776
Year ¥	3	14.312	0.0025
Session	6	6.4486	0.3749
Previous *H. polygyrus* FEC * Previous ivermectin treatment	1	0.3405	0.5595
¥ Variables in the minimal model (P < 0.05)			

2.5 Discussion

In this study I used blood samples from a longitudinal study of wild wood mice, plus data on their *H. polygyrus* treatment and, for some individuals, prior anthelminthic treatment to determine if; 1) *H. polygyrus* infection is associated with being seropositive for WMHV and 2) *H. polygyrus* infection is associated with becoming seropositive for WMHV (they were seronegative for WMHV at the previous capture and have subsequently become seropositive at the next successive capture). I found no association between deworming treatment and being seropositive for WMHV. However, I did find a FEC-dependent effect of treatment; higher previous *H. polygyrus* FECs were associated with increased probability of WMHV infection in mice that were given ivermectin treatments. This is an interesting but counter-intuitive result, as we would expect clearing helminth infections would reduce the

probability of WMHV infection. Additionally, among those that were initially WMHV negative, the probability of becoming infected was not associated with *H. polygyrus* infection or ivermectin treatment. I also found heavy reproductive males had the highest probability being infected with WMHV, which Knowles et al. (2012) hypothesised could be due to specific interactions that occur between reproductive males, such as territorial scent-marking or biting.

Helminth infections are known to have immunosuppressive effects on the host immune system, with many studies showing increased susceptibility to co-infecting microparasitic infections (Actor et al. 1993, Edwards et al. 2005, Dietze et al. 2016). However, our results show no effect of *H. polygyrus* treatment on being, or becoming infected with, WMHV. One reason why we see a different effect of co-infection in our study system compared to other studies may be down to the use of naïve laboratory mice with no history of previous infection challenge in these studies (Actor et al. 1993, Edwards et al. 2005), whereas natural populations will have had previous exposure to other infections which could impact the outcomes of current infections (Cox 2001, Ezenwa et al. 2010). Additionally, the outcomes of co-infections can vary, even among laboratory studies there is some variation in the effect of co-infection on microparasites, for example, a meta-analysis on mouse helminth-malaria co-infection experiments found that co-infection with helminths exacerbated mortality in some studies, but showed protective effects in others by significantly delaying death, while some showed no effect of helminth co-infection on malaria (Knowles 2011). Another study by Scheer et al. (2014) found that prior infection with murine Schistosomiasis had a protective effect against subsequent infection with influenza A virus or pneumonia virus of mice (PVM), with these mice surviving what would normally be lethal doses of influenza A and PVM. Another possibility could be that the effects of co-infection just are not visible over the timescales monitored in this experiment.

I found a surprising effect of prior *H. polygyrus* FECs in treated mice; higher FECs were associated with increased probability of having WMHV at the next capture. This counter intuitive result could be due to less than ideal trapping time scales and the short-lasting effects of ivermectin treatment on *H. polygyrus* infections, meaning mice who were treated have become re-infected with *H. polygyrus*; potentially suggesting that reducing *H. polygyrus* FECs and having them bounce back (as the effect of treatment wears off) can

impact susceptibility to WMHV. It has previously been noted that worm FECs of ivermectin treated mice quickly return to pre-treatment levels (Knowles et al. 2013, Clerc 2017). These results show the effects of co-infection can be varied and unexpected, therefore solidifying the importance of co-infection studies in wild populations to aid our understanding of co-infection dynamics in the wild.

This study suffers from limitations since serological analyses only detect antibodies to WMHV, therefore is no way of knowing if these infections are latent or lytic. WMHV infections start with a lytic phase followed by latent and lifelong infection, which has the potential to reactivate back into the lytic phase during times of immunosuppression (Wu et al. 2000). Since serological assays can only detect if an individual has been infected at some points in its life, these analyses miss any potential interaction whereby *H. polygyrus* causes WMHV to reactivate from latency. To fully understand the effects of helminth infections on WMHV there would need to be a more in-depth analysis to determine if co-infection is associated with lytic WMHV infections.

2.6 Conclusion

In summary, there was a distinct lack of interaction between *H. polygyrus* infection and becoming infected with WMHV. The only interaction that was found was between ivermectin treated mice and prior *H. polygyrus* FEC; higher *H. polygyrus* FECs were associated with increased probability of WMHV infection in treated mice. However, this data potentially suffers from limitations due to only detecting antibodies to WMHV and less than ideal trapping timescales, therefore a more detailed analysis of WMHV infection stages would be needed to fully understand the impact of *H. polygyrus* infection on co-infecting WMHV.

3. Does helminth co-infection influence the likelihood of individuals having a lytic WMHV infection?

3.1 Abstract

Helminth infections are extremely common infections of both humans and wildlife and are well known for modulating the host immune response, hence they are important in co-infection interactions with other parasites within the same host. One group of microparasites of global importance, and for which helminth co-infection may play an important role in their transmission and disease progression, are the gammaherpes viruses (γ-HV). Given the relatively high prevalence of both herpes viruses and helminth infections in many populations, it is not surprising that co-infection between them is common, both in humans and in wildlife. Gamma herpes virus infections typically begin with a lytic phase, which is then followed by a latent and lifelong infection. Here, I analysed samples from wild wood mice, some of which were given deworming treatments, to determine if infection with the helminth *H. polygyrus* increases susceptibility to WMHV infection, or increases the probability of having a lytic WMHV infection. I used open reading frame 73 (*ORF*73) which is one of the few viral genes expressed during viral latency as a marker of latent infection, and open reading frame 50 (*ORF*50) which encodes the viral gene *rta* (replication and transcription activator) and plays a central role in re-activating the virus from latency, to determine whether *H. polygyrus* co-infection affected the likelihood that WMHV infections were in the lytic or latent phase of infection. I found that effective deworming treatments can reduce the probability of an animal also having a WMHV co-infection. However, there was no suggestion that *H. polygyrus* co-infection increases the probability of having a lytic WMHV infection in this wild wood mouse system.

3.2 Introduction

Helminths are ubiquitous throughout nature, infecting many human and most wildlife/livestock populations. They typically cause chronic lifelong infections which are often acquired at an early age, meaning they are a common and persistent part of the host's environment and consequently that of any co-infecting microparasites. Most macro- and microparasites have co-evolved with their hosts over millennia and can influence the host's immune responses and overall fitness. These effects on the host's immune system can then influence susceptibility to, and pathology of, any co-infecting parasites. Such within-host interactions between co-infecting parasites may therefore play a major role in shaping the spread of infectious diseases in both animals and humans (Hotez et al. 2006, Telfer et al. 2008).

3.2.1. *Macro-micro parasite interactions via the host immune response*

Interactions between helminths and microparasites are thought to be driven mainly via the immune system (Cox 2001, Su et al. 2005, Tetsutani et al. 2009, Su et al. 2014). Helminths predominately activate a type 2 (Th2) immune response within the host, promoting the production of cytokines which enable the host to fight off infections by inducing eosinophil differentiation, activating B cells (Chan et al. 1992, Anthony et al. 2007), and inducing alternatively activated macrophages (Weng et al. 2007). However, viruses (and other microparasites) predominantly stimulate a type 1 (Th1) immune response, which is essential for the control of viral infections via $CD8^+$ cytotoxic T-cells and virus neutralising antibodies (Moreau and Chauvin 2010, Palm et al. 2012). These two arms of the immune system are known to be mutually inhibitory. Furthermore, the Th2 immune response simulated by helminth infections can potentially downregulate the Th1 immune response needed to effectively control viral infections resulting in changes in disease susceptibility and progression for a number of microparasitic diseases such as HIV, malaria and TB (Spiegel et al. 2003, Ezenwa et al. 2010, Mulu et al. 2013). Therefore, prior infection with helminths may affect the host's susceptibility to viral infections, and increase disease progression or duration of infection. For example, patients co-infected with both helminths and Hepatitis C virus (HCV) had a decreased HCV specific immune response, when compared to patients with only HCV infections (Farid et al. 2005). In wildlife, voles infected with *Heligmosomum*

mixtum have been found to be more likely to also be infected with Puumala Hantavirus infections (PUUV) (Guivier et al. 2014). Furthermore, helminth infections may not only affect host susceptibility to initial microparasite infection, they can also affect the ability of the host to control or clear the infection once it has taken hold. For example, mice infected with a recombinant vaccinia virus expressing HIV-1, took three weeks longer to clear the virus when co-infected with *Schistosoma mansoni,* compared to when singularly infected with the virus (Actor et al. 1993*).* Likewise, Edwards et al. (2005) found that the liver of mice co-infected with *S. mansoni* and lymphocytic choriomeningitis virus (LCMV) was more susceptible to virus replication, compared to those only infected with LCMV. Hence, prior or ongoing helminth co-infection can alter many aspects of virus infection, host susceptibility and disease progression.

3.2.2. *Deworming to aid in the treatment of major microparasitic infections*

The ubiquity of helminths, coupled with the potentially powerful immune-modulatory effect they exert on their hosts, raises the question of whether the severity and susceptibility of infection with microparasites could be altered using anthelminthic drugs on co-infected individuals (Nacher 2006). However, there are a lot of conflicting results concerning the outcome of helminth removal on co-infecting microparasites (Modjarrad et al. 2005, Walson et al. 2008, Blish et al. 2010). For example, Blish et al. (2010) found that treatment of *Ascaris lumbricoides* in individuals co-infected with HIV-1, results in increased expression of $CD4^+$ cells as well as a trend for decreased HIV-1 RNA levels. Moreover, it was found that in co-infected hosts IL-10 levels were directly correlated with HIV-1 viral load, and negatively correlated with $CD4^+$ cell count, although this was not the case for treated individuals, suggesting that deworming of co-infected individuals may reduce immunosuppression (Blish et al. 2010). Many have published results that support the findings of increased $CD4^+$ cell count, and reduced viral load in treated individuals (Wolday et al. 2002, Kassu et al. 2003, Walson et al. 2008, Lankowski et al. 2014). Despite many studies agreeing with the hypothesis that deworming reduces viral load, increases $CD4^+$ cell count, and ultimately leads to reduced susceptibility and slower disease progression, there are a few that show no, or opposite, results (Modjarrad et al. 2005, Hosseinipour et al. 2007). For example, Modjarrad et al. (2005) found that there was no association between the treatment of helminth infections and reduced HIV viral load in adults in Zambia. Additionally, a study

conducted by Mulu et al. (2013) on HIV-1 infected individuals from Ethiopia with and without helminth co-infections, subsets of which were given a treatment of anthelminthic drugs, found that treatment reduced the HIV RNA levels found in the plasma, but contrary to Blish et al. (2010) there was no reduction in $CD4^+$ cell counts. These studies show that results of deworming can vary, and we therefore need a suitable model system in which to study the effects of deworming on co-infecting parasites. This would allow us to pick apart the interactions between helminths and microparasites and conduct deworming experiments that may be difficult to carry out in human populations.

3.2.3. Wood mice and Gammaherpes viruses

One group of microparasites of global importance, and for which helminth co-infection may play an important role in their transmission and diseases progression, are the gammaherpes viruses (γ-HV). Between 30%-90% of the world's population is infected with at least one species of herpes virus (Shukla and Spear 2001), while more than a quarter of humans are infected with helminths (Pullan et al. 2014). Given the relatively high prevalence of both herpes virus and helminth infections it is not surprising that co-infection between them is common, both in humans and in wildlife (Knowles et al. 2012, Knowles et al. 2013). Gamma herpes virus infections typically begin with a lytic phase, which is typically followed by a latent and lifelong infection (Sunil-Chandra et al. 1992b, Usherwood et al. 1996b, Nash et al. 2001). During viral latency, very few viral genes are expressed therefore allowing it to evade immune control, but also reducing the likelihood of virus transmission to other individuals (Nash et al. 2001). While very few viral genes are expressed during latent infection, open reading frame 73 (*ORF*73) is critical to establish and maintain viral infection in latently infected cells (Fowler et al. 2003, Marques et al. 2003). However, the latent virus has the molecular ability to reactivate into the lytic infection, so re-enabling it to infect new hosts (Wu et al. 2000). The viral gene *rta* (replication and transcription activator) plays a central role in re-activating the virus from latency, and is primarily encoded by open reading frame 50 (*ORF*50) (Liu et al. 2000, Wu et al. 2000, Wu et al. 2001). Investigating the pathogenesis associated with helminth-Herpes virus co-infections in humans can be difficult, so laboratory mouse models are often used to explore these interactions. In the context of helminth co-infection, previous work has explored possible mechanisms of interaction between the nematode *Heligmosomoides polygyrus* and murine herpes virus (MHV-68) in laboratory

mice. Reese et al. (2014) found that infection of laboratory mice with the nematode *Heligmosomoides bakeri* induced expression of the *ORF50* gene in MHV-68, which encodes expression of the *rta* viral protein, causing MHV-68 to reactivate from latency.

Despite these findings in the laboratory setting, questions remain about whether these interactions, measured in laboratory mice, are detectable, or drive the dynamics of infection of these pathogens in their natural host populations. Animals in laboratory settings may be challenged with unnatural combinations of parasite species, or species to whom they are not the natural host. Furthermore, hosts in their natural setting will typically be infected with numerous parasite species, which naturally reside within the host population. Additionally, wild animals will experience other stressors: social interactions, food limitation, other infections, adverse weather, and predation risk- all of which may alter how the host copes with infections and co-infections. This therefore calls into question if the relationship between helminth and microparasite co-infections we see in laboratory animals will be applicable in wild animal populations. It is therefore important that we have a feasible model system to explore the pathogenesis of the herpes viruses in the context of a natural host (Hughes et al. 2010b).

Wood mouse herpes virus (WMHV) is a gamma-herpesvirus, belonging to the family *Herpesviridae* and is most commonly found in wood mice and other murid rodents (Telfer et al. 2007). WMHV is closely related to a number of human herpes viruses (Efstathiou et al. 1990, Virgin et al. 1997, Nash et al. 2001), and the laboratory strain, MHV-68, which is commonly used in laboratory mouse models of infection e.g., (Wu et al. 2000, Wu et al. 2001, Hughes et al. 2010b, Reese et al. 2014, Rolot et al. 2018); WMHV has a nucleotide sequence identity of 85% to MHV-68, and similar pathogenesis (Hughes et al. 2010b). Studies using wild wood mouse populations have found that WMHV infections are higher in reproductively active, heavy males, indicating that male reproductive behaviours could provide an important route of transmission(Knowles et al. 2012).

3.2.4. *Aims and hypotheses*

In this study I investigate the interactions between *H. polygyrus* (the wild-type strain of *H. bakeri*, the laboratory derived strain used by Reese et al. (2014)), and susceptibility to WMHV and its association with a lytic infection in their natural host, the wood mouse

(*Apodemus sylvaticus*), under natural conditions. These wood mice are commonly co-infected with both *H. polygyrus* and WMHV and, as part of an ongoing experiment, some were given anthelmintic (ivermectin and pyrantel) treatments to remove or suppress *H. polygyrus* infections. I examined wood mouse spleen samples from a wild population to test for *ORF73* (expressed during both latent and lytic infection) and *ORF50* (expressed only during lytic infection) gene expression, to determine the presence of latent or lytic WMHV infections, and whether these were influenced by the presence or burden/ fecal egg counts (FEC) of *H. polygyrus* infection, or the treatment status of mice, under natural conditions.

Specifically, I sought to address the following questions:

1) Does current *H. polygyrus* infection or burden affect susceptibility to WMHV infection.

2) Does current *H. polygyrus* infection or burden affect the probability that WMHV infected mice will have latent or lytic infections or affect the quantity of viral *ORF*73 gene expression (used as a proxy measure of viral load).

3) Is there any association between ivermectin treatment and previous *H. polygyrus* infection or FEC, and susceptibility to WMHV infection.

4) Does ivermectin treatment and previous *H. polygyrus* infection or FEC affect the probability that WMHV infected mice will have latent or lytic infections or affect viral *ORF*73 quantities.

3.3 Methods

3.3.1 *Source of samples & data*

Wood mouse spleen samples were collected from wild mouse populations in Falkirk, Scotland from 2013-2014 (Clerc et al. 2018, Clerc et al. 2019a) and 2015-2016 (Sweeny et al. 2019). For the 2013 spleen samples mice were trapped over a 2-month period and sacrificed at first capture; hence these are cross-sectional, resulting in contemporary measures of WMHV and *H. polygyrus* treatment at the same time point for each individual. Here, the measure of *H. polygyrus* infection for these samples is the number of worms in the gut on

dissection. Additionally, morphometric data of the mice were taken such as weight, length, and sex. For the 2014-2016 samples, a longitudinal sampling approach was adopted, whereby all trapped mice were tagged using subcutaneous passive integrated transponder tags so that they could be identified upon recapture. Individuals were assigned to a treatment group on first capture, either deworming treatment (ivermectin and pyrantel) or water as a control. They were then captured daily (Tuesday-Thursday) over ~2-3 week period and given the same treatment during each capture. Around 2 weeks after first capture, animals were sacrificed, and their spleens removed for analysis. Hence, these data are longitudinal, potentially allowing insight into how past infection or treatment status influences subsequent infection. For every animal trapped a faecal sample was collected, along with morphometric details such as weight, length, and sex. Faecal samples from each capture were used to quantify *H. polygyrus* egg count (FEC) by determining the number of *H. polygyrus* eggs in the sample, and number of worms in the gut were counted upon dissection to determine worm burden. For methods on worm egg counts refer to section 2.3.1. Spleen samples were stored in RNA later at -80^{o}C. Eye lens mass was used as a measure of age (Rowe et al. 1985, Clerc et al. 2018, Clerc et al. 2019a).

3.3.2 *Sample selection*

All available spleen samples for 2014 (n=15), 2015 (n=11) & 2016 (n=6) were used in RT-qPCR for WMHV and cytokine gene expression.

As there were many samples for 2013 (n=85), samples were selected to include an even number of male/female mice and *H. polygyrus* status (positive/negative) from the adult population. Any outliers, or those that fell outside the interquartile range in spleen sizes for the population were excluded (Figure 3.1), as very large spleens could be the result of overactive spleens (splenomegaly) from other infections. A total of 32 spleens were selected from 2013.

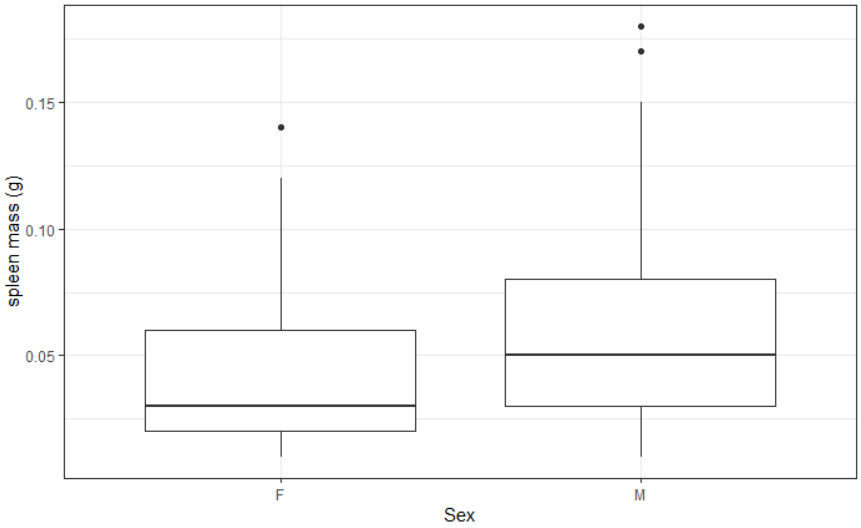

Figure 3.1. Spleen mass (g) for 2013 samples, any spleens with a mass outside the interquartile range were excluded (n=85).

3.3.3 *RNA extraction from spleens*

RNA extraction from spleens (2013) was carried out using RNeasy® PowerLyzer® Tissue & cells kit (Qiagen) according to instructions, including on column DNase 1 treatment. RNA extraction from spleens from 2014 - 2016 was carried out by first homogenising 30mg of spleen sample in lysis buffer (containing mercaptoethanol), before carrying out RNA extraction using RNeasy Mini Kit (Qiagen) including on-column DNase 1 treatment. Optical density of RNA samples was measured to determine RNA concentration before running an agarose gel to ensure there was no DNA contamination. cDNA was reverse transcribed from RNA by first heating 2µg total RNA in the presence of random primers, dNTP's and RNase free water (total volume of 13µl) to 65^{o}C, before snap cooling on ice and adding first strand synthesis buffer plus 0.1M DTT, then heating to 42^{o}C before adding 1µl RTase (SuperScript II) and incubating at room temperature. The mixture was then incubated at 42^{o}C for 2 hours, then inactivated at 70^{o}C. Samples were stored at -20^{o}C until needed.

3.3.4 *RT-qPCR for active/latent WMHV infection*

WMHV presence was examined in spleen samples from 2013-2016 from wild mouse populations in Falkirk, Scotland using PCR and RT-qPCR. cDNA purified from mouse spleen samples was used to amplify WMHV genomic *ORF50* (encodes the key lytic switch protein,

Rta) and *ORF73* (latency associated gene) genes. Primers from MHV-68 were first tried on WMHV known positive samples, after analysing the melt curve from the RT-qPCR analysis it was decided to redesign both *ORF50* and *ORF73* primers using the WMHV genome. These new primers were tested on known WMHV positive samples and the melt curve examined to ensure there was one single peak, an agarose gel was then run to ensure a single band was visible. I also tried different concentrations of $MgCl_2$ and primers in the PCR master mix, and different annealing temperatures for qPCR cycles. However, I was unable to design primers for *ORF*50 that could be used for RT-qPCR in time to use them on samples for this chapter. After spiking samples that had unspecific binding, with the *ORF*50 template I was able to get a perfect melt curve and single band on an agarose gel, suggesting the unspecific binding seen in samples was due to low expression of *ORF*50 in samples. Therefore, this chapter contains only end point PCR results for *ORF*50 as although the primers gave an unspecific melt cure in RT-qPCR they gave a single band on a gel when doing end point PCR. Primers for PCR *ORF*50 (*ORF50*F: 5'- CATCTGAGGACGCGTTCATC- 3' and *ORF50*R: 5'- CAGTGACACATGTTCCAT- 3') generated a 100-bp product (Acc. No. AF105037.1), and *ORF73* (*ORF73*F: 5' ACCATGCCAGGATCTATGYTATTGTGTGT- 3' and *ORF73*R: 5'- CTCCAAAGCATYTACTATTCAA- 3') generated a 157-bp product (Acc. No. GQ169129.1). First PCR was carried out to check the validity of cDNA samples using the murine ribosomal protein L8 gene (acc no. AF091511), using forward primer 5'- ACCAGAGCCGTTGTTGTTGTTGTGG-3' and reverse 5'-AGTTCCTCTTTGCCTTGTACTTGTGG-3'. End point PCR was used to determine the presence of *ORF73* and *ORF50,* the reaction mixture contained 5x PCR buffer, 10x dNTPS (2mM), Taq II Pol (0.8U/µl) and each primer at 10µM each. Additionally, *ORF*73 was analysed by RT-qPCR using the same reaction mixture as for PCR, but with the addition of 10x SYBR green 1. PCR programme was as follows: 35 cycles of 2 min at 95°C, 10s at 94°C, 20s at 60°C, 15s at 72°C, 10s at 75°C, followed by a melt curve analysis of 1 min at 94°C, 30s at 65°C and then 30s at 95°C. The detection limit for *ORF*73 qPCR was 1000 copies.

*ORF*73 copy numbers below 1000 were only included in the analysis if a band was visible on a gel after end point PCR, however these quantities will be less accurate than those above 1000 copies.

3.3.5 *Statistical analysis*

Four analyses were conducted, each examining different aspects of how current or previous *H. polygyrus* infection (presence or absence) or burden/FEC, or ivermectin treatment, could affect WMHV infection. For analyses 1 & 2 helminth burden refers to number of worms in the gut on dissection, for analyses 3 & 4 current and previous FEC refers to number of eggs per gram of faeces collected. Previous *H. polygyrus* refers to FEC/presence in the faeces on first capture, while current *H. polygyrus* refers to FEC /burden/presence (worms in the gut on dissection for analysis 1 and 2, and worms in faeces for analysis 3 and 4) the day they were sacrificed. Each analysis was conducted to address the following questions:

1) Was there any association between current *H. polygyrus* infection or burden and WMHV infection among untreated animals from 2013-2016?

2) Does current *H. polygyrus* infection or burden affect the probability that WMHV infected mice will have a latent or lytic infection or affect viral *ORF*73 quantity among untreated animals from 2013-2016?

3) Is there any association between previous *H. polygyrus* infection or FEC, and WMHV infection among animals from 2014-2016?

4) Does previous *H. polygyrus* infection or FEC affect the probability that WMHV infected mice will have latent or lytic infections or affect viral *ORF7*3 quantity among animals from 2014-2016?

All statistical analysis was performed in R, v. 3.6.1 (R development core team 2011). Here each analysis is explained in more detail:

1) <u>Among untreated animals, is there an association between current *H. polygyrus* infection or burden and WMHV treatment?</u>

Using only untreated individuals from all years (2013-2016), I first analysed whether *H. polygyrus* infection was associated with the likelihood of WMHV infection (regardless of whether latent or lytic). I used a binomial GLM with WMHV infection (factor: yes or no), based on expression of any viral gene (*ORF*73 and/or *ORF*50) as the response variable and *H. polygyrus* (Hp) infection (factor: yes or no):

ORF73/ORF50 ~ Hp infection + eye lens mass * sex + year,

or *H. polygyrus* (Hp) burden (continuous):

ORF73/ORF50 ~ Hp burden + eye lens mass * sex + year,

as the key predictor of interest. In each case I controlled for the following covariates: eye lens mass (continuous), host sex (factor, male or female) and year (Factor: 2013 -2016). In all cases, full models were created for each explanatory variable above, plus a sex*lens mass interaction, since previous work has suggested that older males are more likely to be WMHV-positive than any other demographic group (Knowles et al. 2012). Models were then simplified by backwards elimination of non-significant terms, initially with a cut-off at ($p<0.07$), starting with interaction terms, until a minimal model was reached with all terms $p<0.05$.

2) Among untreated animals is current *H. polygyrus* infection or burden associated with the probability of WMHV infections being lytic/latent or orf73 copy numbers?

To determine the effect of *H. polygyrus* infection or burden on the probability of WMHV infections being lytic/latent (using only untreated individuals from 2013-2016), an analysis was carried out using only WMHV positive individuals (those with either *ORF73* and/or *ORF50* expression). Among those animals, individuals that were positive for *ORF*50 expression were assumed to harbour lytic infection; those without were assumed to harbour latent infection. Again, I used binomial GLM's with lytic WMHV infection (factor: yes or no), based on expression of *ORF*50 as the response variable, and as the key predictors of interest either *H. polygyrus* infection (factor: yes or no):

ORF50 ~ Hp infection + eye lens mass * sex + year,

or *H. polygyrus* burden (continuous):

ORF50 ~ Hp burden + eye lens mass * sex + year,

Next, to determine if *H. polygyrus* infection or burden affects the quantity of *ORF*73 gene expression (a proxy for viral load), an analysis was carried out using *ORF*73 RT-qPCR data

which had been normalised against the mouse L8 housekeeping gene. This assay had a detection limit of 1000 copies, therefore any quantities below this are less accurate, however all had bands of the correct size on an agarose gel, and so were included even if below the detection limit. I used Gaussian GLM's with *ORF*73 (logged, continuous) as the explanatory variable and as the key predictor of interest either *H. polygyrus* infection (factor: yes or no):

*ORF*73 ~ Hp infection + eye lens mass * sex + year+ *ORF50* expression,

or *H. polygyrus* burden (continuous):

*ORF*73 ~ Hp burden + eye lens mass * sex + year + *ORF50* expression.

Each model was tested for the effect of the covariates and interactions listed above, including *ORF50* expression and simplified as above.

3) Does previous ivermectin treatment (or current *H. polygyrus* infection), and previous *H. polygyrus* infection/burden, affect susceptibility to WMHV?

This analysis uses only individuals from years where ivermectin treatments were given under a longitudinal study design (2014-2016) to determine if ivermectin treatment/previous *H. polygyrus* infection (presence/absence) is associated with WMHV infection. Crammers V test of association was used to determine whether current *H. polygyrus* infection (presence/absence) and ivermectin treatment were associated (v=0.73, p<0.001). Since current *H. polygyrus* treatment and ivermectin treatment are highly associated (those that were treated did not have current *H. polygyrus* infections), alternative versions of these models were explored. These involved either current *H. polygyrus* infection or ivermectin treatment being included as the key predictor of interest in the model, along with previous *H. polygyrus* infection, and models were compared using AIC. Since previous *H. polygyrus* infection status (determined from faecal sample collected at the time of sacrifice) was not associated by Crammer v test of association with either treatment (v=1.17, p=0.38) or current *H. polygyrus* infection (v=0.29, p=0.12) it could be included in both models. As assessed by AIC, having ivermectin treatment in the models (AIC: 33.64) provided a better description of the data than having current *H. polygyrus* in the models (AIC: 37.54). Therefore, binomial GLM's were used with WMHV infection (factor:

yes or no), based on expression of any viral gene (*ORF*73 and/or *ORF*50) as the response variable with ivermectin treatment (factor: yes or no) and previous *H. polygyrus* infection (factor: yes or no) as the key predictors of interest:

*ORF*73/*ORF50* ~ Previous Hp infection + Ivermectin treatment + eye lens mass * sex + year + *ORF50* expression.

To determine if current or previous *H. polygyrus* FEC is associated with WMHV infection, again I used binomial GLM's with WMHV infection (factor: yes or no) as the response variable with current *H. polygyrus* FEC (continuous) and previous *H. polygyrus* FEC (continuous) as the key predictors of interest:

*ORF*73/*ORF50* ~ Previous Hp FEC + current Hp FEC + eye lens mass * sex + year + *ORF50* expression.

Again current *H. polygyrus* FEC and previous *H. polygyrus* FEC were not correlated by Pearson test of correlation (r=-0.1, p=0.4) so could both be included in the model.

Each model was tested for the effect of the covariates and interactions listed above, with the addition of *ORF*50 expression and simplified as above.

4) Does ivermectin treatment (or current *H. polygyrus* infection), and previous *H. polygyrus* infection/FEC affect the probability of WMHV infections being lytic/latent or *ORF73* copy numbers?

Again, this analysis used only individuals from years where ivermectin treatments were given (2014-2016), but now restricted to only those animals with a detectable WMHV infection. As above, AIC values were used to determine if ivermectin treatment or current *H. polygyrus* infection (presence/absence) should be used in the models. The model containing treatment was better (AIC: 37.80) than the model containing current *H. polygyrus* infection (AIC: 37.94), so binomial GLM's were used with lytic WMHV infection (factor: yes or no, based on *ORF*50 presence/absence), with ivermectin treatment (factor: yes or no) and previous *H. polygyrus* infection (factor: yes or no) as the key predictors of interest:

ORF50 ~ Previous Hp infection + Ivermectin treatment + eye lens mass * sex + year.

To determine if current or previous *H. polygyrus* FEC is associated with lytic WMHV infection, again binomial GLM's were used with lytic WMHV infection (factor: yes or no) as the response variable with current *H. polygyrus* FEC (continuous) and previous *H. polygyrus* FECFEC (continuous) as the key predictors of interest:

ORF50 ~ Previous Hp FECFEC + current Hp FEC + eye lens mass * sex + year.

Again current *H. polygyrus* FEC and previous *H. polygyrus* FEC were not correlated by Pearson test of correlation (r=-0.2, p=0.3), so could both be included in the model.

AIC values were also used to determine whether current *H. polygyrus* infection or treatment should be included in the *ORF*73 expression (quantity) models. The AIC value for the model with current *H. polygyrus* infection was marginally better (AIC: 141.94) than the model containing ivermectin treatment (AIC: 141.98). As in question (2) I used Gaussian GLM's with *ORF*73 (logged, continuous) as the explanatory variable with current *H. polygyrus* infection (factor: yes or no) and previous *H. polygyrus* infection (factor: yes or no):

ORF73 ~ Previous Hp infection + current Hp infection + eye lens mass * sex + year + *ORF5*0,

and a second model with current *H. polygyrus* FEC (continuous) and previous *H. polygyrus* FEC (continuous):

ORF73 ~ Previous Hp FEC + current Hp FEC + eye lens mass * sex + year *ORF5*0,

as the key predictors of interest. Each model was tested for the effect of the covariates and interactions listed above, with the addition of *ORF50* expression and simplified as above.

3.4 Results

A total of 61 spleen samples were analysed for expression of viral *ORF*73 (one of the main genes expressed during latent infection) and *ORF*50 (encodes the key lytic switch protein, Rta), from 2013 -2016. Across all years *ORF*73 expression was detected in 45.9% of samples, while *ORF*50 expression was detected in 24.6% of samples. Additionally, 63.9% of individuals had a *H. polygyrus* infection (Table 3.1). From this table we can see that there is the possibility of a 20% false negative rate as we have 3 samples that were ORF50 positive but ORF73 negative.

Table 3.1. Number of individuals with different combinations of *ORF*73, *ORF*50 and *H. polygyrus* (Hp) positive or negative.

*ORF*50	*ORF*73 Negative		*ORF*73 Positive	
	Hp +	Hp -	Hp +	Hp -
Negative	15	15	13	3
Positive	2	1	9	3

3.4.1 *Among untreated animals is there an association between current H. polygyrus infection or burden and WMHV infection?*

There was no effect of current *H. polygyrus* infection on the likelihood of also having WMHV (regardless of active or latent) (X^2_1=1.33, p=0.25; Table 2A). However, year (X^2_3=16.57, p<0.001) and a lens mass*sex interaction (X^2_1=5.10, p=0.02) remained in the model (Table 3.2A, Figure 3.2). There was also no effect of current *H. polygyrus* burden on the likelihood of having WMHV (X^2_1=0.48, p=0.49; Table 2B), but year and a lens mass* sex interaction remained in the model as above (Table 3.2B).

Table 3.2. Factors affecting susceptibility to WMHV in a wild wood mouse population. Model containing: A) *H. polygyrus* presence/absence and B) *H. polygyrus* burden (total worms in the gut on dissection). Models were simplified by backwards stepwise elimination. For variables not in the minimal model, statistics are those at the point they left the model.

A Variable	df	X^2	p
Lens mass*Sex ¥	1	5.1049	0.0239
Lens mass	-	-	-
Sex	-	-	-
Year ¥	3	16.5735	0.0009
H. polygyrus infection	1	1.3273	0.2493
B Variable	df	X^2	p
Lens mass*Sex ¥	1	5.1049	0.0239
Lens mass	-	-	-
Sex	-	-	-
Year ¥	3	16.5735	0.0009
H. polygyrus burden	1	0.4813	0.4878
¥ indicates terms retained in the final model (p<0.05)			

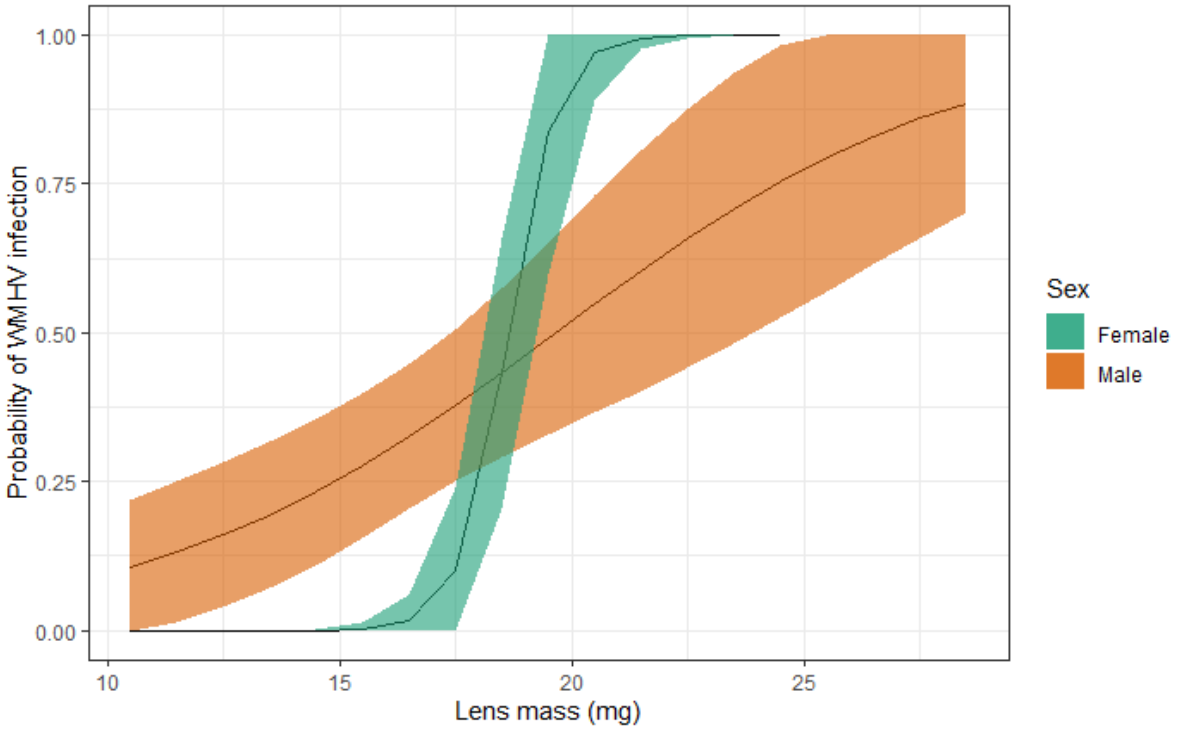

Figure 3.2. The association between eye lens mass, for males and females and the probability of having a WMHV infection plotted for the year 2013 (n=32).

3.4.2 *Among untreated animals is current H. polygyrus infection or burden associated with the probability of WMHV infections being lytic, or ORF73 copy numbers?*

Among those with a WMHV infection there was no effect of *H. polygyrus* infection (X^2_1=0.44, p=0.51; Table 3.3A) or *H. polygyrus* burden (X^2_1=1.36, p=0.24; Table 3.3B) on the probability of having a lytic WMHV infection. Additionally, there was no effect of current *H. polygyrus* infection on *ORF*73 copy numbers (X^2_1=1.65, p=0.20; Table 3.4A). However, there was an effect of current *H. polygyrus* burden on *ORF*73 copy numbers (X^2_1=8.72, p=<0.01), with higher burdens resulting in increased *ORF*73 expression (Table 3.4B, Figure 3.3).

Table 3.3. Factors affecting the probability of WMHV infected wild wood mice having an active infection. Model containing: A) *H. polygyrus* presence/absence and B) *H. polygyrus* burden (total worms in the gut on dissection). Models were simplified by backwards stepwise elimination. For variables not in the minimal model, statistics are those at the point they left the model.

A Variable	df	X^2	p
Lens mass*Sex	1	0.3068	0.5797
Lens mass	1	0.6037	0.4372
Sex	1	0.0119	0.9131
Year	3	5.5681	0.1346
H. polygyrus infection	1	0.4404	0.5070
B Variable	df	X^2	P
Lens mass*Sex	1	0.0488	0.8252
Lens mass	1	0.0923	0.7613
Sex	1	0.0033	0.9539
Year	3	5.5681	0.1346
H. polygyrus burden	1	1.3621	0.2432
¥ indicates terms retained in the final model (p<0.05)			

Table 3.4. Factors affecting viral *ORF*73 expression in WMHV infected wild wood mice. Model containing: A) *H. polygyrus* presence/absence and B) *H. polygyrus* burden (total worms in the gut on dissection). Models were simplified by backwards stepwise elimination. For variables not in the minimal model, statistics are those at the point that factor left the model.

A Variable	df	X^2	p
Lens mass*Sex	1	0.5125	0.4741
Lens mass	1	3.0037	0.0831
Sex	1	0.9562	0.3281
Year	3	4.0593	0.2551
H. polygyrus infection	1	1.6465	0.1994
Orf50 expression	1	0.3336	0.5636
B Variable	df	X^2	P
Lens mass*Sex	1	0.0012	0.9727
Lens mass	1	0.0508	0.8217
Sex	1	1.1793	0.2775
Year	3	2.5084	0.4738
H. polygyrus burden ¥	1	8.7197	0.0031
Orf50 expression	1	0.0728	0.7872
¥ indicates terms retained in the final model (p<0.05)			

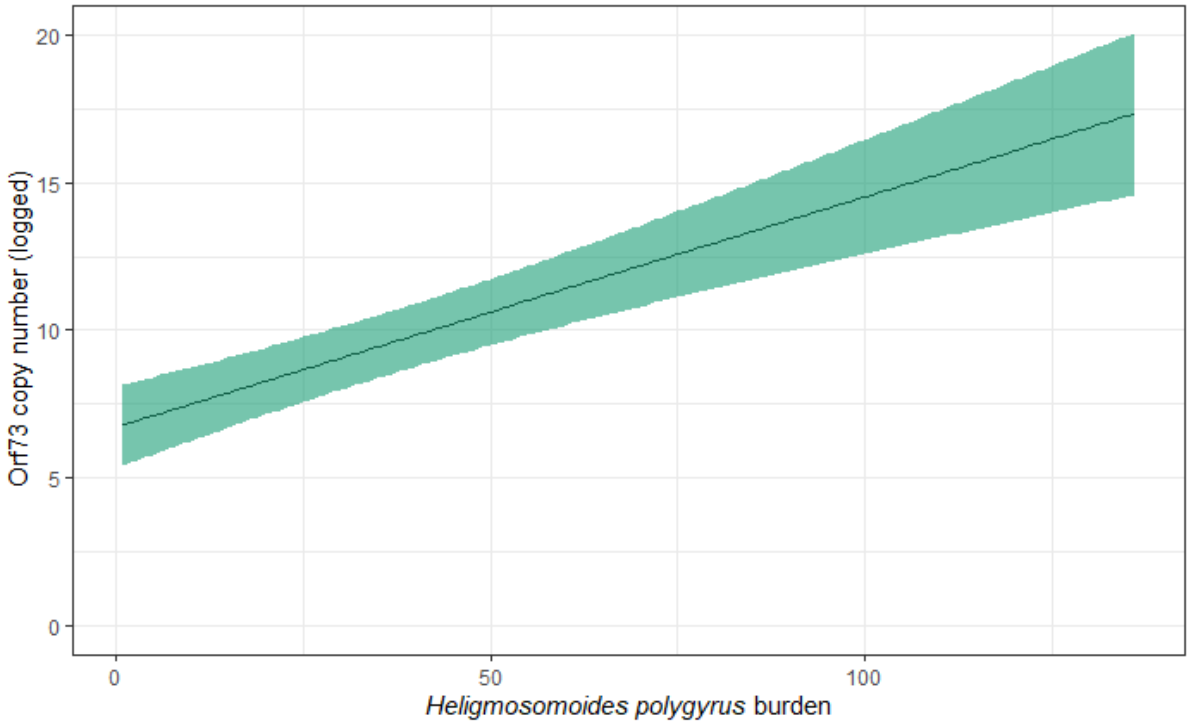

Figure 3.3. The association between *Heligmosomoides polygyrus* infection burden and viral *ORF*73 expression (logged copy number) (±SEM) in wood mouse herpes virus (WMHV) infected wild wood mice. Increasing *H. polygyrus* burdens are associated with higher *ORF*73 gene expression (n=64).

3.4.3 *Does previous deworming treatment or previous H. polygyrus infection/*FEC *affect susceptibility to WMHV?*

There was a significant negative association between deworming treatment and being infected with WMHV (X^2_1= 7.37, p=0.007; Table 3.5A, Figure 3.4). However, there was no association between previous *H. polygyrus* infection and WMHV infection (X^2_1=1.28, p=0.26) (table 3.5a). Additionally, neither previous (X^2_1=0.87, p=0.35) nor current (X^2_1=0.001, p=0.98) *H. polygyrus* FEC were associated with WMHV infection (Table 3.5B).

Table 3.5. Factors affecting WMHV infection in wild wood mice. Model containing: A) previous *H. polygyrus* presence/absence and ivermectin treatment and B) current and previous *H. polygyrus* FEC (from feceal egg counts). Models were simplified by backwards stepwise elimination. For variables not in the minimal model, statistics are those at the point they left the model.

A Variable	df	X^2	p
Lens mass*Sex	1	1.2275	0.2679
Lens mass	1	1.8914	0.1690
Sex	1	1.7472	0.1862
Year	3	0.3240	0.8504
Previous *H. polygyrus* infection	1	1.2835	0.2573
Ivermectin treatment ¥	1	7.3739	0.0066
B Variable	df	X^2	P
Lens mass*Sex ¥	1	4.2121	0.0401
Lens mass	1	-	-
Sex	1	-	-
Year	3	0.1290	0.9376
Previous *H. polygyrus* FEC	1	0.8672	0.3517
Current *H. polygyrus* FEC	1	0.0009	0.9767
¥ indicates terms retained in the final model ($p<0.05$)			

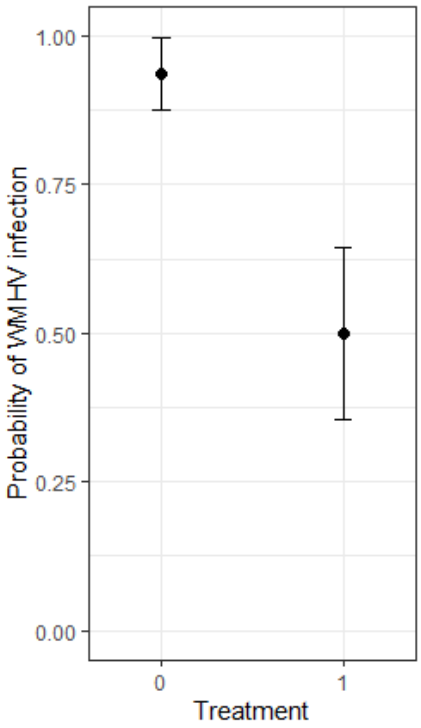

Figure 3.4. The association between treated (Ivermectin + Pyrantel) (untreated (0), treated (1)) and wood mouse herpes virus infection (WMHV) in a wild wood mouse population (n= 32). Mice given deworming treatments were less likely to have a WMHV infection.

3.4.4 *Does previous ivermectin treatment or previous H. polygyrus infection/*FEC *affect the probability of WMHV infections being lytic/latent, or ORF73 copy numbers?*

There was no association between ivermectin treatment (X^2_1= 0.37, p=0.54) or previous *H. polygyrus* treatment (X^2_1= 0.40, p=0.53) and the probability of having active WMHV infections (Table 6A). Additionally, there was no association between current *H. polygyrus* FEC (X^2_1= 3.02, p=0.08) or previous *H. polygyrus* FEC (X^2_1= 1.50, p=0.22) and the probability of having active WMHV infections (Table 3.6B).

The amount of viral *ORF*73 expression was not associated with deworming treatment (X^2_1= 0.14, p=0.83) or previous *H. polygyrus* infection (X^2_1= 3.31, p=0.07) (Table 3.7A). Additionally, there was no association with current *H. polygyrus* infection (X^2_1= 0.04, p=0.83) or previous *H. polygyrus* FEC (X^2_1= 0.01, p=0.94) (Table 3.7B).

Table 3.6. Factors affecting the probability of WMHV infected wild wood mice having an active infection. Model containing: A) previous *H. polygyrus* presence/absence and ivermectin treatment and B) previous *H. polygyrus* FEC (from feceal egg counts) and ivermectin treatment. Models were simplified by backwards stepwise elimination. For variables not in the minimal model, statistics are those at the point that factor left the model.

A Variable	df	X^2	p
Lens mass*Sex	1	0.1558	0.6930
Lens mass	1	1.7342	0.1879
Sex	1	0.5961	0.4401
Year	3	3.8227	0.1479
Previous *H. polygyrus* infection	1	3.3128	0.0687
Current *H. polygyrus* infection	1	0.1441	0.8336
Orf50 expression	1	1.0886	0.2968
B Variable	df	X^2	P
Lens mass*Sex	1	0.0187	0.8912
Lens mass	1	1.7342	0.1879
Sex	1	0.7275	0.3937
Year	3	3.8227	0.1479
Previous *H. polygyrus* FEC	1	0.0052	0.9427
Deworming treatment	1	0.0441	0.8337
Orf50 expression	1	0.7296	0.3930
¥ indicates terms retained in the final model (p<0.05)			

Table 3.7. Factors affecting viral *ORF*73 expression in WMHV infected wild wood mice. Model containing: A) previous *H. polygyrus* presence/absence and current *H. polygyrus* presence/absence and B) previous *H. polygyrus* FEC (from feceal egg counts) and Deworming treatment. Models were simplified by backwards stepwise elimination. For variables not in the minimal model, statistics are those at the point they left the model.

A Variable	df	X^2	p
Lens mass*Sex	1	1.1458	0.2844
Lens mass	1	0.4914	0.4833
Sex	1	0.2924	0.5887
Year	3	4.5624	0.1022
Previous *H. polygyrus* infection	1	0.4027	0.5257
Ivermectin treatment	1	0.3677	0.5442
B Variable	df	X^2	P
Lens mass*Sex	1	2.7364	0.0981
Lens mass	1	1.0512	0.3052
Sex	1	0.3512	0.5534
Year	3	4.1991	0.1225
Previous *H. polygyrus* FEC	1	1.5005	0.2206
Current *H. polygyrus* FEC	1	3.0186	0.0823
¥ indicates terms retained in the final model ($p<0.05$)			

3.5 Discussion

In this study I used spleen samples collected from wild wood mice, plus data on their *H. polygyrus* treatment and, for some animals, prior anthelmintic treatment to determine if; 1) *H. polygyrus* infection is associated with susceptibility to WMHV, 2) *H. polygyrus* infection is associated with WMHV infections being lytic or affects control of WMHV gene expression (i.e. does it increase/decrease *ORF*73 gene expression), 3) previous *H. polygyrus* infection or ivermectin treatment is associated with susceptibility to WMHV, and 4) previous *H. polygyrus* infection or ivermectin treatment is associated with WMHV infections being lytic or affects control of WMHV gene expression? This study suggests that treatment with ivermectin (therefore reduced FECs) is associated with a decrease in susceptibility to WMHV infection, and that higher *H. polygyrus* burdens are associated with increased levels of *ORF*73 gene expression, a proxy for viral load of WMHV. Furthermore, older females (age based on lens mass) were more likely to have a WMHV infection than older males. However,

H. polygyrus infection (current or previous) had no detectable effect on either the likelihood of an individual having WMHV infection, or on the likelihood of a WMHV-positive animal having a lytic infection.

In contrast to the results found by Knowles et al. (2012), who found that WMHV infection probability was highest in reproductively active heavy male mice, this study found that older females were more likely to have a WMHV infection. However, I was unable to include reproductive status due to small sample sizes. While this could mean that older females in our system are more susceptible than males to WMHV; alternatively, it could be that older females are no more likely to become infected than males, however when they do become infected they are less able to suppress viral RNA expression in the spleen to below detectable levels.

3.5.1. *Reduced probability of WMHV in ivermectin treated wood mice*

The reduced likelihood of WMHV infection in individuals treated with ivermectin suggests that *H. polygyrus* and WMHV could be engaging in some sort of within-host interaction, whereby individuals infected with *H. polygyrus* are more likely to be infected with WMHV, and hence their removal by ivermectin treatment removes this interaction and reduces the likelihood of being infected with WMHV. *H. polygyrus* is known to have suppressive effects on the host immune system with implications for susceptibility to, and progression of, infections by other microparasites. For example, Su et al. (2014) used laboratory mice co-infected with *H. polygyrus* and *Salmonella enterica* serovar typhimurium, and found that mice with *H. polygyrus*– *S. typhimurium* co-infections had a higher mortality rate and more severe pathology than those with single *S. typhimurium* infections. One method of immune modulation includes the secretion of excretory-secretory (ES) products from adult worms (Hewitson et al. 2009). Additionally*, H. polygyrus* infection predominantly initiates a Th2 immune response (Mohrs et al. 2005), which has been linked to the downregulation of the microparasite fighting Th1 arm of the immune response (Salgame et al. 2013). Therefore, immune perturbation caused by chronic helminth infection could be a confounding factor in increased susceptibility to WMHV. Our results support those found by Guivier et al. (2014) who studied wild vole populations and found a positive association between the nematode *Heligmosomum mixtum* and Puumala Hantavirus infections (PUUV), whereby those infected

with *H. mixtum* were more likely to become infected with PUUV. Moreover, Ezenwa et al. (2010) illustrated the importance of helminth infection in the facilitation of microparasitic infections. They found that helminth infection in wild free-ranging buffalo caused immunosuppression which facilitated the infection of buffalo with tuberculosis (TB); most notably, in populations without helminth infections TB was predicted to fail to invade the system (Ezenwa et al. 2010).

It has previously been suggested that removal of helminths by deworming could potentially reverse the inhibitory effects of the Th2 response on the Th1 response (Bentwich et al. 1995, Bentwich et al. 1999). Elias et al. (2001) hypothesized that reduced vaccine efficiency against TB in countries where helminth infections are common, is due to the strong Th2 bias caused by helminth infection. They found that Th1 responses (INF-γ) were down regulated in individuals with concurrent helminth infections, however this was shown to be reversed in individuals that received deworming treatments (Elias et al. 2001). Our data shows that individuals treated with ivermectin are less likely to have a detectable WMHV infection, which could potentially be due to a reversal of the inhibitory effects of the Th2 response. However, analysis of Th1/Th2 cytokines would be needed to determine if Th1 cytokines are down regulated in co-infected individuals, and if this is reversed in ivermectin treated individuals. Analysis of the cytokine responses of the animals in this study are presented in the next chapter.

3.5.2. *Higher H. polygyrus burdens are associated with higher ORF73 gene expression*

Additionally, our results suggest that *H. polygyrus* burden also affects the host's ability to control WMHV, as higher *H. polygyrus* burdens were associated with higher *ORF*73 gene expression. Increased helminth burden has been known to exacerbate the negative effects of helminth infection (Goater and Ward 1992, Lowrie et al. 2004), for example in mice, increasing helminth burdens have been associated with increased host weight loss (Lowrie et al. 2004). Additionally, Goater and Ward (1992) found that toads with higher worm burdens were smaller in size, had higher mortality rates, and reduced dietary intake. All these consequences of increased helminth burden could potentially exacerbate the effects of co-infection.

3.5.3. *There is no association between H. polygyrus co-infection and lytic WMHV infections*

Since WMHV causes latent lifelong infections which can re-activate if the host is immunocompromised, we may expect to find that those co-infected with *H. polygyrus* are less able to keep latent WMHV infections under control, if the Th2 immune response caused by *H. polygyrus* is down regulating the Th1 immune response needed to control viral infections. However, I show that in our wild mouse populations *H. polygyrus* infection is not associated with individuals having a lytic WMHV infection. These results are in contrast to those found by Reese et al. (2014) who used laboratory mice models infected with *H. bakeri* and MHV-68. They found that *H. bakeri* initiated the Th2 immune response, resulting in the release of interleukin (IL) 4, which activates pathways resulting in the expression of *ORF50* and consequently re-activating MHV-68 from latency. However, it has recently been called into question the relevance of these highly artificial studies for real world wild animals (Beura et al. 2016a, Reese et al. 2016). Wild animals, over the course of their lives are faced with a diverse array of parasite infections, most of these will not be in isolation, or indeed be limited to the two species co-infections seen in laboratory co-infection studies. Additionally, wild animals will experience other stressors: social interactions, food limitation, other infections, adverse weather, and predation risk- all of which make the immune functions of wild mice more variable than those of laboratory strains (Abolins et al. 2011). For example, Abolins et al. (2011) found that wild caught mice (*Mus musculus*), had greater immune function, more avid antigen-specific immune responses, and a greater overall activation of immune cells than laboratory-bred mice. Furthermore, laboratory studies often use unnatural combinations of host and parasites, and so their findings may be of limited relevance for combinations that have coevolved together over prolonged time periods. Taking these factors into account may explain why our results in wild populations differ from those in laboratory model studies. Our results highlight how important it is to conduct these experiments in wild populations and could explain why results seen in the laboratory cannot always be reproduced in the field. However, I had relatively low sample numbers which could also be a limiting factor when trying to detect these types of interactions, thus it may be that a larger set of samples would need to be analysed to fully rule out the interactions seen in laboratory mice.

3.6 Conclusion

These results show that mice treated with the anthelmintic drugs ivermectin and pyrantel, were less likely to be infected with WMHV, while higher *H. polygyrus* burdens were associated with reduced ability to control WMHV infections, hinting at potential immune mediated interactions between *H. polygyrus* and WMHV in our wild mouse system. However, in contrast to previous laboratory studies I found no association between *H. polygyrus* infection or ivermectin treatment and lytic WMHV infections, highlighting the importance of conducting these studies in wild naturally infected populations.

4. Is helminth-viral co-infection associated with reduced levels of Th1 gene expression?

4.1 Abstract

At some point in their lives most animals, and many human populations will be co-infected with multiple parasite species. Co-infections involving helminths and microparasites are of importance as they are dealt with differently by the immune system, and these different arms of the immune system may be antagonistic to each other, such that the presence of one parasite may affect the ability of the host to mount an effective immune response against the other. Helminths tend to induce a Type 2 (Th2) immune response, whereas viruses (and other microparasites) induce a type 1 (Th1) immune response. Here, I use a wild wood mouse population, and RT-qPCR for some Th1 and Th2 cytokine genes, to determine if co-infection with *H. polygyrus* results in the downregulation of the anti-viral Th1 arm of the immune system. I found that in this wild wood mouse population mice with current or previous *H. polygyrus* co-infections had reduced Type 1 cytokine gene expression (*Tnf*-α, IL-6 and *Tgf*-β) compared to those with only WMHV infections. Additionally, there was no evidence of Th2/immunoregulatory cytokines being upregulated in response to *H. polygyrus* infection. Overall, these results suggest that there could be a lag between upregulation of Th2 cytokines and the downregulation of Th1 cytokines which our trapping time scales were not able to capture. Additionally, it could be other immune mechanisms/cytokines which are responsible for the downregulation of the Th1 cytokines.

4.2 Introduction

Most animals, and indeed many human populations are co-infected with both helminth and microparasite species at some point in their lives (Petney and Andrews 1998, Cox 2001). These co-infections are of particular importance as they activate mutually exclusive arms of the immune system, and these arms may be antagonistic to each other, such that the presence of one parasite may alter the immune response towards another. Helminths tend to induce a Type 2 (Th2) immune response, whereas viruses (and other microparasites) induce a type 1 (Th1) immune response (Mosmann and Sad 1996). The Th2 immune response is characterised by the proliferation and activation of T-cells that secrete Interleukin (IL) 4, IL-5, IL-9, IL-10 and IL-13 (Anthony et al. 2007). These cytokines all play a role in mounting an effective immune response against helminth infections. In contrast, microparasites tend to induce a pro-inflammatory Th1 immune response which is essential for the control of viral infections via $CD8^+$ cytotoxic T-cells and virus neutralising antibodies, and results in increased levels of IL-2, IL-23, tumor necrosis factor (TNF)-α, and interferon (IFN)- γ (Anthony et al. 2007, Alves et al. 2009, Moreau and Chauvin 2010). Recently, some Th1 responses have been split into a 'new' T-cell subset which is distinct from Th1 cells, called Th17 cells, which are crucial for controlling microparasitic infections (Milner et al. 2008, Dienz et al. 2012). They are known to secrete IL-17 along with pro-inflammatory cytokines IL-6 and TNF-α (Kolls and Lindén 2004, Langrish et al. 2005, Anthony et al. 2007). TGF- β has been found to help in the differentiation of these Th17 cells, giving it an important role in the activation of the pro-inflammatory response (Veldhoen and Stockinger 2006).

Cytokines released during a Th2 immune response have their own important functions; IL-13 plays a major role in the development of fibrosis (Chiaramonte et al. 1999, Cheever et al. 2000), IL-5 regulates eosinophil production (De'Broski et al. 2000) which act as killer cells (Capron and Capron 1992) and as helper cells for protective antibody production (De'Broski et al. 2000), and IL-4 protects against a severe, lethal inflammatory condition in mice (Fallon et al. 2000), and is important for helminth expulsion (Urban et al. 2000), and IL-6 controls the differentiation of monocytes and macrophages, increases B-cell IgG production and

promoted the Th2 response via IL-4 (Velazquez-Salinas et al. 2019). Additionally, some Th2 cytokines, such as IL-10, play a role in limiting damage to the host by its own immune system: by regulating the Th2 and Th1 responses during helminth infection, IL-10 can minimise immunopathology (Flores-Villanueva et al. 1996, Hoffmann et al. 2000). Interferons (IFNs) can initiate a diverse set of anti-microbial functions. IFN-γ is produced mainly by natural killer (NK) cells and T cells during infection with virus/bacteria/protozoa, and results in a number of downstream effects including inducing antimicrobial/autophagy response in macrophages, upregulation of MHC class I and II molecules, and activates T cell responses through enhancement of phagocytosis and antigen presentation (Newport et al. 1996, Gutierrez et al. 2004, Schroder et al. 2004, Fabri et al. 2011, Rhein et al. 2015). For example, Rhein et al. (2015) found that mice given murine IFN-γ 24 hours before or after infection with mouse adapted Ebola virus were protected against lethal challenges with the virus, reduced morbidity and reduced viral titres in serum. The Th2 responses aimed at controlling helminth infections have the potential to downregulate Th1 cytokines such as INF-γ, IL-12 and TNF-α, which are essential for promoting effector mechanisms involved in fighting viral infections (Mosmann and Sad 1996). Hence, individuals co-infected with Th2-initiating parasites (such as helminths) may be more susceptible to, and less able to control, co-infecting microparasites such as viruses (Spiegel et al. 2003, Ezenwa et al. 2010).

4.2.1 *The effect of Helminth infection on co-infecting microparasites*

As an example of helminth-virus interactions, Reese et al. (2014) found that helminth infection, characterized by induction of the cytokine IL-4, caused on-going murine γ-herpesvirus infection to become reactivated from latency *in vivo*. IL-4 promoted viral replication by binding to and acting on a viral promoter to induce expression of the viral latent-to-lytic switch gene (open reading frame 50 (*ORF*50)), and blocked the antiviral effects of INF-γ (Reese et al. 2014). Similarly, Actor et al. (1993) found that mice co-infected with *Schistosoma mansoni* and a recombinant vaccinia virus expressing the HIV-1 protein gp120, could take as much as 3 weeks longer to clear the virus, compared to mice only infected with the vaccinia virus. Additionally, when spleen cells taken from mice infected with only vaccinia virus were stimulated with gp120 they produced higher INF- γ and IL-2, but lower IL-4 responses than the mice infected with *S. mansoni* only. However, those

infected with both the vaccinia virus and *S. mansoni* produced lower INF- γ and IL-2 responses when challenged with gp120 than the vaccinia virus alone, but produced no IL-4 response above the non-specific response displayed by *S. mansoni* only infected mice (Actor et al. 1993). These results show that cytokines induced by helminth infections can affect a host's ability to respond to co-infecting viruses by suppressing the anti-viral Th1 arm of the immune system.

As suggested by the previous examples, most of our understanding of the immune response during co-infections has come from highly controlled laboratory settings involving laboratory mice kept in abnormally clean "specific pathogen free" (SPF) barrier facilities (Babayan et al. 2011, Beura et al. 2016b). Additionally, laboratory mice have limited genetic diversity, receive a constant supply of food and water and have far less experience with infectious disease than free-living animals would naturally acquire (Pedersen and Babayan 2011, Maizels and Nussey 2013, Cadwell 2015). This is very different to what wild animals (and humans) experience. Wild animals have much more genetic diversity, variable amounts of food and water, diverse environmental conditions, and may be infected (or have been infected) with multiple pathogen species (Cox 2001, Ezenwa et al. 2010, Telfer et al. 2010). Hence, it is unsurprising that laboratory mice raised in SPF conditions have been found to have immune systems more closely related to that of neonatal humans than of adult humans (Beura et al. 2016b), whereas, 'dirty' pet store and wild mice had more differentiated memory T cells in lymphoid and non-lymphoid tissue, which more closely matches those of adult humans (Beura et al. 2016b). Moreover, animals in the wild are frequently infected with both Th1 stimulating micro-parasites and Th2 stimulating macro-parasites, which can lead to immune driven within-host parasite interactions (Ezenwa and Jolles 2011), potentially influencing the abundance, distribution, and dynamics of each parasite species (Pedersen and Fenton 2007). Laboratory studies also often use unnatural host-parasite combinations, potentially resulting in differently with-in host-parasite interactions than would be seen in the natural host. Therefore, to fully understand the host immune response, how it drives co-infection dynamics and allow results to better capture the adult human response, we may need to move more towards 'dirty' or wild mice for immunological studies.

4.2.2 *Deworming to control major microparasitic diseases*

Additionally, the possibility that helminth infection can exacerbate micro-parasitic infections has called into question whether mass deworming could be beneficial in controlling major viral, bacterial and protozoal diseases (Nacher 2006, Gerns et al. 2012). For example, Malla et al. (2006) found that individuals infected with the nematode *Ascaris lumbricoides* had lower levels of Th1 cytokines and higher levels of Th2 cytokines, compared to uninfected controls. Additionally, albendazole treatment of HIV-1 and *A. lumbricoides* co-infected individuals resulted in reduced IL-10 levels, compared to untreated individuals (Blish et al. 2010). Another study by Elias et al. (2001) found that when blood samples of people treated with albendazole, so reducing their worm burdens, were stimulated with Tuberculin purified protein derivative (PPD- which is used as a diagnostic aid in the detection of Mycobacterium tuberculosis infection) they showed an improvement in T-cell proliferation and increased INF- γ production. In contrast, helminth infection has been shown to have a protective effect against malaria infection, with co-infected children having lower *Plasmodium falciparum* parasite densities than those with only malaria infections (Briand et al. 2005, Lemaitre et al. 2014). These studies show that the effect of helminth removal on the immune response, and co-infecting micro-parasites can be hard to predict. We therefore need a suitable model system, which can easily be manipulated with deworming treatments, to fully understand the effect of helminth removal on the host immune response.

4.2.3 *Hypotheses*

The murine intestinal nematode, *Heligmosomoides bakeri,* triggers a highly polarized type 2 immune response within the host (Gause et al. 2003). The type 2 immune response aimed at *H. polygyrus* infections is known to downregulate the type 1 immune response needed to control WMHV infections. This chapter explores whether *H. polygyrus* infection alters levels of type 1 cytokine expression which are aimed at controlling WMHV infections, under natural field conditions. Through semi-quantitative RT-qPCR for a number of type 1 and type 2 cytokine genes from wild wood mouse spleens used in chapter 3, I will determine if *H. polygyrus* co-infection, ivermectin treatment and/or WMHV infection (latent or lytic) are

associated with increased, or decreased gene expression levels of type 1 or type 2 cytokines. Based on previous work, it is expected that *H. polygyrus* infection could increase the expression of type 2 cytokines (IL-5 and IL-10), and consequently reduce expression of type 1 cytokines (*Tnf*-α, *Inf*-γ, IL-6 and *Tgf*- β) aimed at WMHV infection. *Tgf*- β expression has been shown to be important in the pro-inflammatory immune response, as it helps with the differentiation of the highly inflammatory Th17 cells. Additionally, it is expected that treatment with ivermectin could be associated with increased Th1 cytokine gene expression, and reduced Th2 cytokine gene expression.

4.3 Methods

For details on source of wild rodent spleen samples and RNA extraction refer to methods sections 3.3.1 – 3.3.3 in chapter 3.

4.3.1 *PCR for immune gene*

Relative RT-qPCR was used to compare Th1 and Th2 cytokine expression using the Pfaffl equation (Pfaffl 2001), using the murine ribosomal protein L8 gene as a reference gene (acc no. AF091511) forward primer 5'-ACCAGAGCCGTTGTTGTTGTTGTGG-3' and reverse 5'-AGTTCCTCTTTGCCTTGTACTTGTGG-3'. The Pfaffl equation allows relative quantification to be calculated between groups based on the relative expression of a target gene versus a reference gene, when target and reference genes have different primer efficiencies. For example, to determine if co-infected mice have reduced Th1 responses compared to those infected only with WMHV, or if ivermectin treated mice have increased Th1 cytokine gene expression compared to untreated mice. cDNA purified from mouse spleen samples was used to amplify the mouse genes associated with a Th1 or Th2 immune response (Table 4.1)(Acc. No. GQ169129.1). Primer pairs that had previously been designed by the Pedersen group at the University of Edinburgh were tested in the same way as in chapter 3. I also tested primer pairs that had been designed for laboratory mice, however none gave a suitable melt curve. The efficiency of primer pairs was first checked by running a standard curve, to ensure all efficiencies were between 1.9 and 2.1. PCR reaction mixture contained 5x PCR buffer, 10x dNTPS (2mM), 10x SYBR green 1, Taq II Pol (0.8U/µl) and each primer at 10µM each. PCR programme was as follows: 2 min at 95°C, then 35 cycles of 10s at 94°C,

20s at 60°C, 15s at 72°C, 10s at 75°C, followed by a melt cure analysis of 1 min at 94°C, 30s at 65°C and then 30s at 95°C, run on a LightCycler480.

Table 4.1. Primer pairs used for RT-qPCR

Gene	Primer pair	Product length
IL-5	*IL5F*: 5' – GTGTAGCGAGGAGAGACGGAGGA- 3' *IL5R*: 5' – CCCACTCTGTACCCATCACGGCA -3'	80bp
IL-6	*IL6F*: 5' – GCAAGAGACTTCCATCCAGTTGCC – 3' *IL6R*: 5' – AAGCCTCCGACTTGTGAAGTGGT – 3'	146bp
I*nf*-γ	*InfgF*: 5' – TCTCCTGCTTCGGCCTAGCTCTG – 3' *InfgR*: 5' – AGGCTTTCGATGAGTGTGCCGTG – 3'	117bp
Tnf-α	*TnfaF*: 5' – GCACAGAAAGCATGATCCGCGAC – 3' *TnfaR*: 5' – CCACGAGCAGGAACGAGAAGAGAC – 3'	120bp
Tgf-β1	*Tgfb1F*: 5' – GGTGGACCGCAACAACGCAATCT – 3' *Tgfb1R*: 5' – CCGCCCGGAACAGCAACGG – 3'	122bp
IL-10	*IL10F: 5' – TTTAAGGGTTACTTGGGTTGC* – 3' *IL10R: 5' – TCAAATGCTCCTTGATTTCTG* – 3'	109bp

4.3.2 *Statistical analysis*

All statistical analysis was performed in R, v. 3.6.1 (R development core team 2011).

1) Does *H. polygyrus* co-infection downregulate the expression of cytokines aimed at WMHV infection?

To determine if expression of each cytokine is associated with infection status among untreated mice from 2013-2016, I used Gaussian GLM's with relative expression of each cytokine (square root transformed), as the response variable, and infection status (4-level factor: uninfected, co-infected, WMHV only, *H. polygyrus* only) as the explanatory variable of interest:

Cytokine ~ Infection status + eye lens mass * sex + year

The infection status variable used 'current' *H. polygyrus* infection, collected at the same time point as the cytokine data, determined by presence of worms in the gut on dissection. In each case I controlled for the following covariates: eye lens mass (continuous) as a proxy for host age (Clerc 2018; 2019), host sex (factor, male or female) and year (factor: 2013-2016). Interactions were also included to test for age specific sex effects (eye lens mass * sex). In all cases, full models were created for each response variable above, and then

models were simplified by backwards elimination of non-significant terms initially with a cut off at ($p<0.07$), starting with interactions, to obtain a minimal model with all terms $p<0.05$.

2) Do Th1 cytokine expression levels change depending on WMHV infection status (lytic or latent)?

To determine if cytokine levels differ between WMHV infection stages (i.e. latent/lytic), only untreated mice with WMHV infections from 2013-2016 were included in this analysis. Gaussian GLM's were used with relative expression of each cytokine (square root transformed), as the response variable, to determine if WMHV status (factor: infected latent, infected lytic, based on detectable levels of *ORF*73 and *ORF*50; see Chapter 3), and *H. polygyrus* co-infection (factor: co-infected, WMHV only) are associated with levels of cytokine expression (relative to L8) among WMHV infected mice:

Cytokine ~ WMHV status + Hp co-infection + eye lens mass * sex + year.

Again, the *H. polygyrus* co-infection variable uses current *H. polygyrus* infection determined by presence of worms in the gut on dissection. In each case I controlled for the same covariates and simplified as above.

3) Does *H. polygyrus* co-infection or deworming treatment alter the expression of cytokines aimed at WMHV infection?

For this analysis only mice from 2014-2016 were included, as in these years some mice were also given treatments. Crammers V test of association was used to determine whether current *H. polygyrus* treatment (presence/absence) and ivermectin treatment were associated ($v=0.73$, $p<0.001$). Since current *H. polygyrus* infection status (determined by eggs in faecal samples at the time of sacrifice) and treatment (individuals were either given ivermectin and pyrantel or a control of water repeatedly over a period of ~2 weeks) are highly correlated only one can be included in the models. Hence 2 models were constructed for each cytokine, one containing current *H. polygyrus* infection status, and the other containing ivermectin treatment. These models used Gaussian GLM's with relative expression of each cytokine (square root transformed), as the response variable, to determine if either current *H. polygyrus* infection (factor: yes or no):

Cytokine ~ current Hp infection + eye lens mass * sex + year,

or ivermectin treatment (factor: yes or no) and previous *H. polygyrus* infection (factor: yes or no):

Cytokine ~ Ivermectin treatment + current Hp infection + eye lens mass * sex + year,

are associated with levels of cytokine expression (relative to L8). The two models were compared for each cytokine using AIC to determine which model best explained the data. Since previous *H. polygyrus* infection status (determined from faecal sample collected at the time of sacrifice) was not associated by Crammer v test of association with either treatment (v=1.17, p=0.38) or current *H. polygyrus* infection (v=0.29, p=0.12) it could be included in both models.

4) Do higher *H. polygyrus* FECs alter the expression of cytokines aimed at WMHV infection?

Again, for this analysis only mice from 2014-2016 were included, as these years had data on current and previous *H. polygyrus* FECs. A set of Gaussian GLMs were constructed with relative expression of each cytokine (square root transformed), as the response variable, with current and previous *H. polygyrus* FEC (continuous) as the main explanatory variables of interest:

Cytokine ~ current Hp FEC + previous Hp FEC + eye lens mass * sex + year.

Current and previous *H. polygyrus* FEC were not correlated (r_{25}=0.03, p=0.90) so could both be included in the model. In all models I controlled for the same covariates and simplified as above as above.

4.4 Results

A total of 61 spleen samples were collected from wild wood mice, some of which had been given deworming treatments and which had corresponding co-infection, and WMHV treatment data (table 4.2).

Table 4.2. Number of wild wood mice in each treatment group for ivermectin + pyrantel treated and untreated (2014-2016), and 2013 (all untreated).

Treatment	Treated (2014-2016)	Untreated (2014-2016)	Untreated (2013)
WMHV only	5	0	2
Co-infected	1	15	8
Worms only	3	1	11
Uninfected	3	0	12

4.4.1 *Does H. polygyrus co-infection downregulate the expression of cytokines aimed at WMHV infection?*

Overall, only *Tnf*- α gene expression was found to be significantly associated with infection status (X^2_3=11.01 , p= 0.012) (Table 4.3). Animals with WMHV-only infections had higher gene expression compared to co-infected, *H. polygyrus* only and uninfected animals (Figure 4.1). This model also included significant effects of year (X^2_3= 14.80, p= 0.002) and a lens mass * sex (X^2_1=5.96, p= 0.015) interaction. Although not significant infection status (X^2_3= 4.45, p= 0.217) was the last term to drop out of the model for *Inf*-γ and, as for *Tnf*- α (another Th1 cytokine) expression was highest for WMHV only infected mice (Figure 4.2).

No other infection-related factors were found to be significant for any of the other cytokines (Table 4.3). However, there were significant differences among years for IL-6 (X^2_3= 12.62, p= 0.006), IL-10 (X^2_3= 10.81, p= 0.013) and *Tgf*- β (X^2_3= 42.07, p<0.0001) with the addition of a lens mass*Sex interaction (X^2_1= 5.87, p=0.015) (Figure 4.3).

Table 4.3. Factors affecting cytokine expression levels (relative to L8) in a wild wood mouse population. Models were simplified by backwards stepwise elimination. For variables not in the minimal model, statistics are those at the point that factor left the model.

		A. IL-5		B. IL-6		C. IL-10	
Variable	df	X^2	p	X^2	p	X^2	p
Lens mass*Sex	1	2.0704	0.1502	0.0710	0.7898	0.0740	0.7857
infection status	3	3.9545	0.2664	2.5613	0.4643	1.0022	0.8007
Lens mass	1	0.6089	0.4352	0.1913	0.6619	0.1516	0.6970
Sex	1	0.8336	0.3612	0.1246	0.7241	0.3416	0.5589
Year	3	5.5854	0.1336	12.622	0.0055 ¥	10.809	0.0128 ¥
		A. *Tnf*-α		B. *Inf*-γ		C. *Tgf*-β	
Variable	df	X^2	p	X^2	p	X^2	p
Lens mass*Sex	1	5.9582	0.0146 ¥	3.0851	0.7901	5.8700	0.0154 ¥
infection status	3	11.005	0.0117 ¥	4.4487	0.2169	0.6560	0.8836
Lens mass	1	-	-	0.1193	0.7298	-	-
Sex	1	-	-	0.0108	0.9173	-	-
Year	3	14.800	0.0020 ¥	4.4432	0.2174	42.0680	<0.0001 ¥
¥ Variables in the minimal model (P < 0.05)							

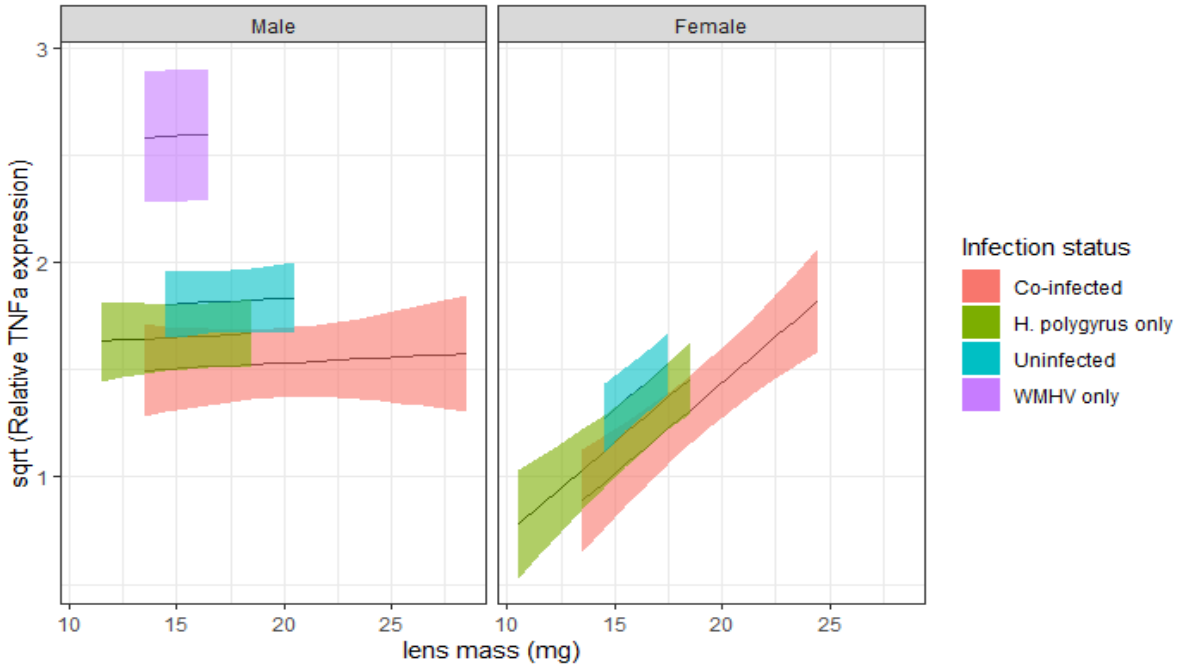

Figure 4.1. Association between *Tnf*-α gene expression and lens mass (mg), sex and infection status among untreated wild wood mice in 2013 (n=32). There were no females with only WMHV. Overall, there was a significant increase in *Tnf*-α gene expression in WMHV-only mice compared to the other infection categories.

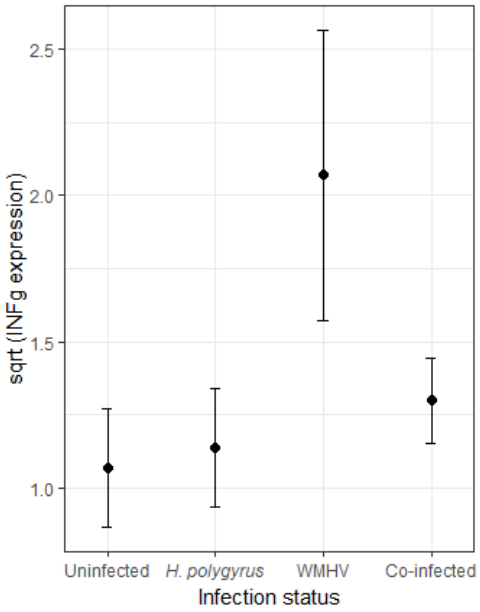

Figure 4.2. Association been *Inf*-γ gene expression and infection status among untreated wild wood mice (n=64). Infection status is not significant and is shown for illustrative purposes only.

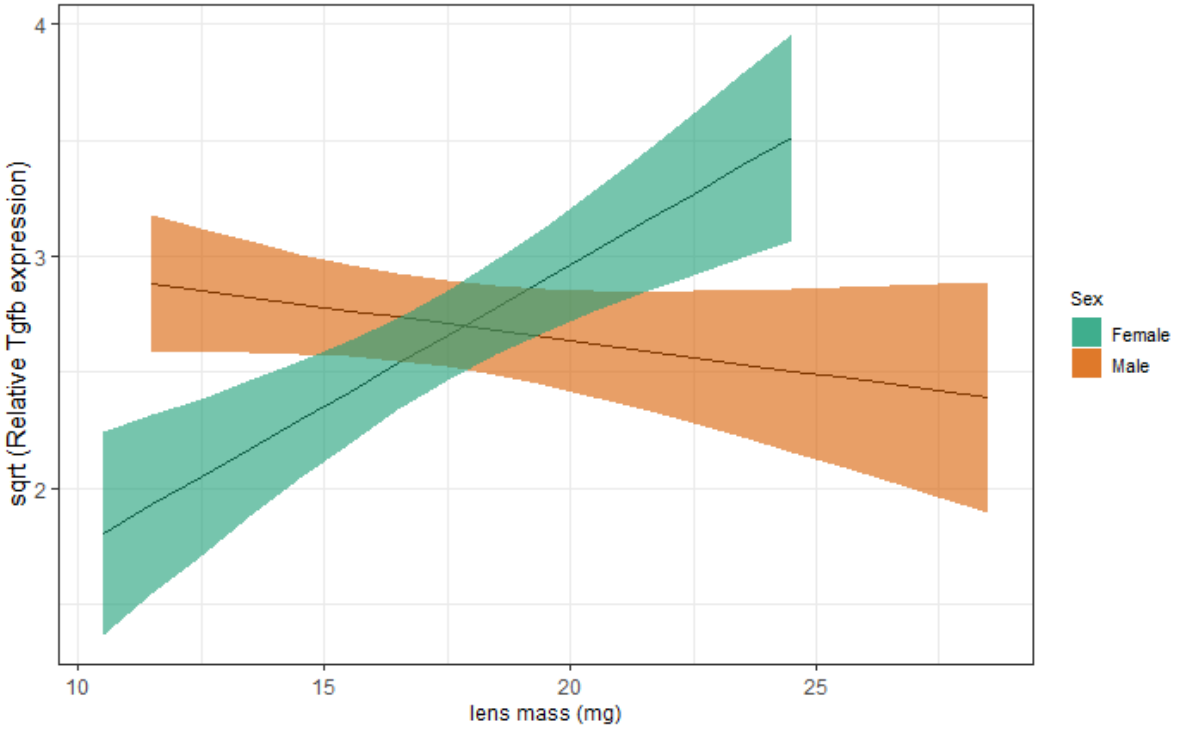

Figure 4.3. Association been *Tgf*- β gene expression, lens mass and sex among untreated wild wood mice for 2013 (n=32). Older females are more likely to have higher *Tgf*-β gene expression than males.

4.4.2 *Do Th1 cytokine expression levels change depending on WMHV* infection status *(lytic or latent)?*

Overall, none of the cytokines showed significantly different levels of expression between lytic or latent WMHV infections (Table 4.4). However, there were significant differences among years for IL-6 (X^2_3=9.24, p=0.03), IL-5 (X^2_3=11.32, p=0.01), and IL-10 (X^2_3=11.41, p=0.01). Additionally, *Tgf*-β had significant differences among years (X^2_3=21.60, p<0.0001) plus a lens mass * sex interaction (X^2_1=4.40, p=0.04), *Inf*-γ had only a significant lens mass * sex interaction (X^2_1=6.67, p=0.01), and *Tnf*-α had significant differences among years (X^2_3=15.37, p=0.002), infection status (X^2_1=7.05, p=0.008) and a lens mass sex interaction (X^2_3=11.34, p<0.001) (Table 4.4).

Table 4.4. Factors affecting cytokine expression levels in wild wood mice infected with WMHV. Models were simplified by backwards stepwise elimination. For variables not in the minimal model, statistics are those at the point they left the model.

		A. IL-5		B. IL-6		C. IL-10	
Variable	df	X^2	p	X^2	p	X^2	p
Lens mass*Sex	1	2.9782	0.0844	2.2865	0.1305	3.1063	0.0780
Treatment	1	1.5107	0.2190	0.5789	0.4468	1.0459	0.3065
Lens mass	1	0.2607	0.6097	0.0205	0.8860	2.3438	0.1258
Sex	1	1.6297	0.2017	0.0057	0.9400	0.5397	0.4625
Year	3	11.323	0.0101 ¥	9.2359	0.0263 ¥	11.407	0.0097 ¥
WMHV infection status	1	2.6179	0.1057	0.0009	0.9767	3.4039	0.0650

		D. *Tnf*-α		E. *Inf*-γ		F. *Tgf*-β	
Variable	df	X^2	p	X^2	p	X^2	p
Lens mass*Sex	1	11.341	0.0008 ¥	6.6689	0.0098 ¥	4.4001	0.0360 ¥
Treatment	1	7.0503	0.0079 ¥	1.0868	0.2972	0.1529	0.6958
Lens mass	1	-	-	-	-	-	-
Sex	1	-	-	-	-	-	-
Year	3	15.365	0.0015 ¥	2.7799	0.4268	21.598	<0.0001 ¥
WMHV infection status	1	0.0043	0.9480	1.0565	0.3040	0.2388	0.6251

¥ Variables in the minimal model (P < 0.05)

4.4.3 *Does H. polygyrus co-infection or deworming treatment alter the expression of cytokines aimed at WMHV infection?*

AIC values were used to determine whether treatment or current *H. polygyrus* infections should be in each model. The models containing treatment were better than models containing current *H. polygyrus* infection for *Tnf*-α, IL-6 and *Tgf*-β. However, models containing current *H. polygyrus* infection were better for IL-5, *Inf*-γ and IL-10 (Table 4.5).

Table 4.5. AIC values for GLM's containing either treatment or current *H. polygyrus* infection (Presence/absence). Numbers in bold indicate the model with the lowest AIC.

	Model AIC	
Cytokine	Deworming treatment	Current *H. polygyrus*
Tnf-α	**43.35**	45.40
IL-6	**120.79**	122.09
Tgf-β	**71.21**	73.26
IL-5	69.10	**68.80**
Inf-γ	82.24	**80.73**
IL-10	42.49	**39.98**

Overall, neither treatment nor current *H. polygyrus* infection were significantly associated with any cytokines (Table 4.6). However previous *H. polygyrus* infection (presence/absence) (X^2_1= 5.61, p= 0.02) and year (X^2_2= 6.00, p=0.05) were significantly associated with levels of IL-6 gene expression; individuals with previous *H. polygyrus* infections had reduced IL-6 expression (Table 4.6; Figure 4.4). Similarly, previous *H. polygyrus* infection (X^2_1= 10.26, p= 0.001) was associated with reduced expression of *Tgf*-β (Table 4.6; Figure 4). In addition, year (X^2_2= 12.25, p= 0.002), and a lens mass * sex interaction (X^2_1= 5.56, p= 0.02) were also significantly associated with *Tgf*-β expression- in males expression decreases with increasing lens mass, while in females expression increases with increasing lens mass (Table 4.6; Figure 4.5). All other models had either no significant variables (*Inf*-γ), year (IL-5: X^2_2= 7.51, p= 0.02;

IL-10: X^2_2= 6.36, p= 0.04), or a significant lens mass effect (*Tnf*-α: X^2_1= 6.28, p= 0.01) (Table 4.6).

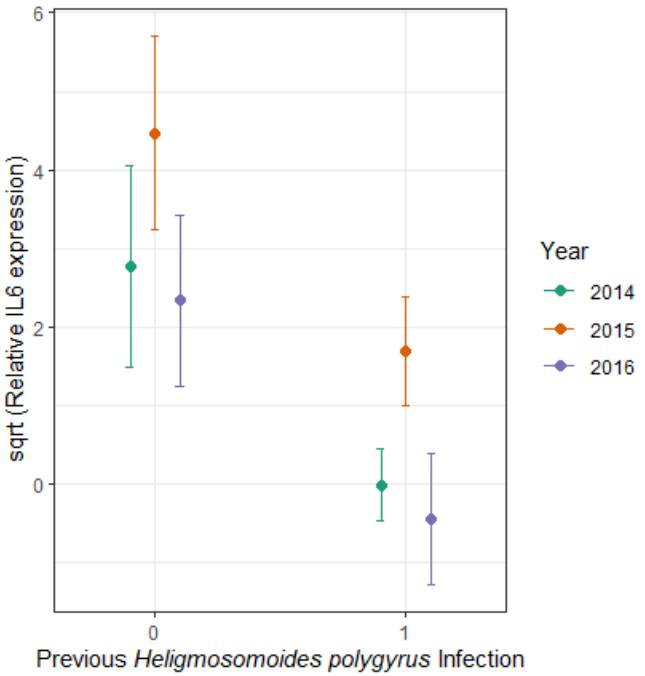

Figure 4.4. Relationship between IL-6 expression, and previous *H. polygyrus* infection status (presence(1)/absence(0)) and year in a wild wood mouse population (n=32). Wood mice with previous *H. polygyrus* infections had lower expression of IL-6.

Table 4.6. Factors affecting cytokine expression levels in a wild wood mouse population, some of which have been given ivermectin treatments. Models were simplified by backwards stepwise elimination. For variables not in the minimal model, statistics are those at the point they left the model.

		Tnf-α (AIC: 44.46)		IL-6 (AIC: 125.74)		*Tgf*-β (AIC: 82.79)		
Variable	df	X^2	p	X^2	p	X^2	p	
Lens mass*Sex	1	0.1538	0.6949	0.3320	0.5645	5.5552	0.0184	¥
Ivermectin treatment	1	2.3599	0.1245	2.3216	0.1276	2.1603	0.1416	
Lens mass	1	6.2846	0.0122 ¥	2.1771	0.1401	-	-	
Sex	1	0.0114	0.9151	0.0012	0.9722	-	-	
Year	2	0.5889	0.7450	5.9950	0.0499 ¥	12.245	0.0022	¥
Previous *H. polygyrus*	1	0.0609	0.8052	5.6138	0.0178 ¥	10.260	0.0014	¥
		IL-5 (AIC: 67.53)		*Inf*-γ (AIC: 83.45)		IL-10 (AIC: 40.55)		
Variable	df	X^2	p	X^2	p	X^2	p	
Lens mass*Sex	1	0.4307	0.5117	0.0117	0.9140	2.8108	0.0936	
Current *H. polygyrus*	1	0.4054	0.5243	1.4134	0.2345	1.5969	0.2063	
Lens mass	1	2.1523	0.1424	0.0970	0.7555	1.9439	0.1632	
Sex	1	0.4758	0.4903	1.6409	0.2002	0.4539	0.5005	
Year	2	7.5077	0.0234 ¥	1.9781	0.3719	6.3620	0.0415	
Previous *H. polygyrus*	1	0.0165	0.8977	0.7418	0.1869	0.4982	0.4803	
¥ Variables in the minimal model (P < 0.05)								

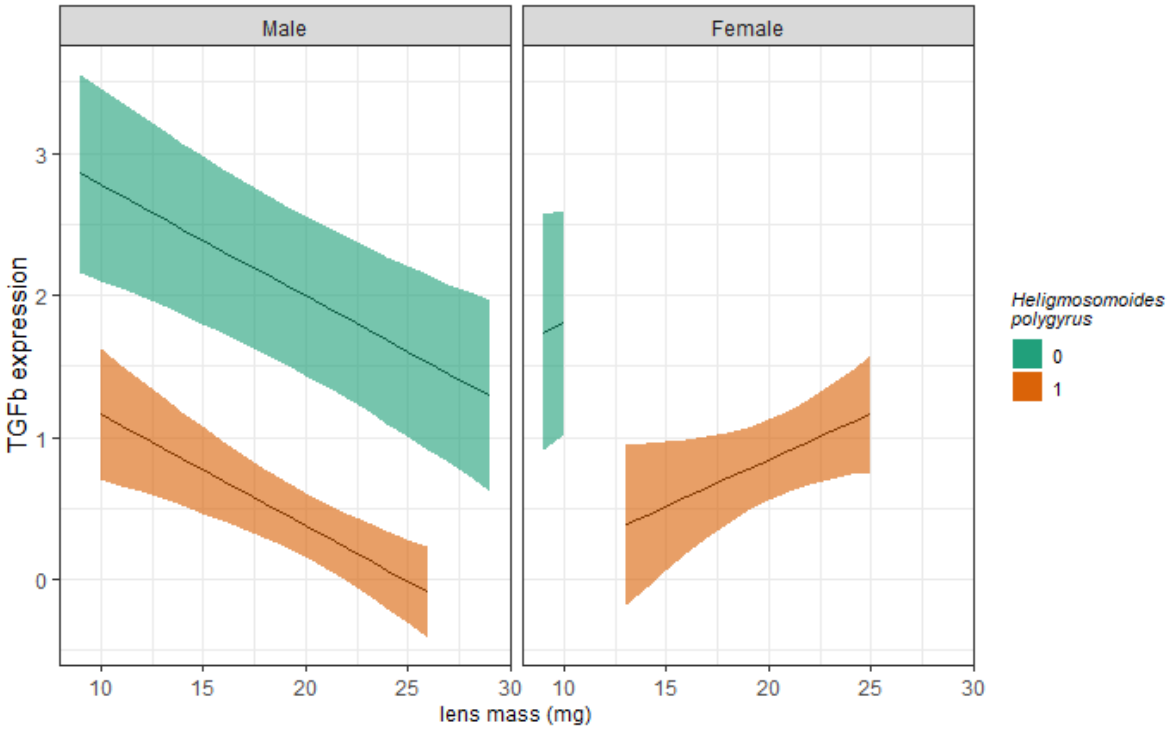

Figure 4.5. Relationship between *Tgf*-β gene expression (relative to L8), lens mass (proxy for age), previous *H. polygyrus* infection status (presence(1)/absence(0)) and year (2014) in a wild wood mouse population (n=15).

4.4.4 *Do higher H. polygyrus* FEC*s alter the expression of cytokines aimed at WMHV infection?*

Overall, neither current nor previous *H. polygyrus* FEC was associated with levels of any cytokine expression (Table 4.7). The only variables that remained in the models were year for *Tgf*-β (X^2_2=8.82, p=0.01), IL-5 (X^2_2=7.51, p=0.02). and IL-10 (X^2_2=6.36, p=0.04), and lens mass for TNF-α (X^2_1=6.28, p=0.01).

Table 4.7. Factors affecting cytokine expression levels in a wild wood mouse population, some of which have been given ivermectin treatments. Models were simplified by backwards stepwise elimination. For variables not in the minimal model, statistics are those at the point they left the model.

		Tnf-α			IL-6			*Tgf*-β		
Variable	df	X^2	p		X^2	p		X^2	p	
Lens mass*Sex	1	0.2393	0.6247		1.1957	0.2742		1.9636	0.1611	
Current *H. Polygyrus* FEC	1	0.4598	0.4977		0.5676	0.4512		0.0747	0.7845	
Lens mass	1	6.2846	0.0122	¥	2.7051	0.1000		2.6581	0.1030	
Sex	1	0.0592	0.8078		0.1505	0.6981		1.5204	0.2146	
Year	2	0.9473	0.6227		5.7144	0.0574		8.8209	0.0122	¥
Previous *H. polygyrus* FEC	1	0.3581	0.5563		0.8828	0.3475		0.5730	0.4491	
		IL-10			*Inf*-γ			IL-5		
Variable	df	X^2	p		X^2	p		X^2	p	
Lens mass*Sex	1	2.4334	0.1188		0.0339	0.8538		0.2199	0.6391	
Current *H. Polygyrus* FEC	1	0.0139	0.9061		0.2581	0.6115		2.6007	0.1068	
Lens mass	1	1.9439	0.1632		0.0623	0.8029		1.1581	0.2819	
Sex	1	0.6619	0.4159		0.6409	0.2002		0.6000	0.4386	
Year	2	6.3620	0.0415	¥	2.1710	0.3377		7.5077	0.0234	¥
Previous *H. polygyrus* FEC	1	1.6766	0.1954		0.0001	0.9943		0.1664	0.6834	
¥ Variables in the minimal model (P < 0.05)										

4.5 Discussion

In this study spleen samples from wild wood mice, plus data on their *H. polygyrus* and WMHV infection status and, for some animals, prior anthelminthic treatment was used to answer the following questions: 1) Does *H. polygyrus* co-infection downregulate the expression of cytokines aimed at WMHV infection? 2) Do Th1 cytokine expression levels change depending on WMHV infection status (lytic or latent)? 3) Does *H. polygyrus* co-infection or deworming treatment alter the expression of cytokines aimed at WMHV infection? 4) Do higher *H. polygyrus* FECs alter the expression of cytokines aimed at WMHV infection? This study suggests that expression of some Th1 cytokines are downregulated in *H. polygyrus* co-infected mice, compared to mice with WMHV only infections. In mice with current *H. polygyrus* infections both TNF-α and INF-γ (not statistically significant), showed reduced expression compared to WMHV only infected mice. Additionally, mice that had previous *H. polygyrus* infections had reduced type 1 immune responses, IL-6 and TGF-β, compared to mice with only WMHV infections. In contrast there was no association between treatment, WMHV status (latent or lytic), current *H. polygyrus* FECs or previous *H. polygyrus* FECs and expression of any cytokines.

4.5.1. *Reduced Th1 cytokine expression in co-infected wood mice*

One of the main findings of this study shows that animals with *H. polygyrus* co-infections have reduced Th1 cytokine expression compared to animals with only WMHV infections. Data from this chapter showed that *Tnf*-α expression was higher in mice with WMHV-only infections, however in mice with *H. polygyrus* co-infections expression was reduced to levels similar to those with *H. polygyrus* only infections, or uninfected mice. Although not statistically significant, *Inf*-γ also showed to be slightly higher in WMHV-only mice, again with expression levels falling in co-infected mice. These results suggest that co-infection with *H. polygyrus* can downmodulate these pro-inflammatory Th1 cytokines, which are essential for controlling virus infections. Other studies have found similar results, for example, infection of mice with the protozoan parasite *Cryptosporidium parvum* results in higher levels of INF-γ, however co-infection with *Heligmosomoides bakeri* (the laboratory species of the wild *H. polygyrus* found to infect wood mice) inhibits INF-γ secretion (Bednarska et al. 2008). Another study found that the larval stage of the helminth *Ascaris*

suum downregulated the immune response to *Vaccinia* virus (VACV), with reduced numbers of circulating $CD4^+$ T-cells producing INF-γ in mice co-infected with both *A. suum* and VACV, compared to those only infected with VACV (Gazzinelli-Guimarães et al. 2017). The Th1 cytokine TNF-α are known to be particularly important in controlling microparasitic infections and have been found in increased quantities in the spleens of MHV-68 infected mice (Sarawar et al. 1996). For instance, TNF-α plays a major role in the maturation of dendritic cells after challenge with adenovirus (Trevejo et al. 2001). Furthermore, the cytokine IL-17 which is produced by Th-17 cells (Langrish et al. 2005) mediates tissue inflammation by inducing IL-6 and TNF-α, however infection with *H. polygyrus* has been found to suppress the production of IL-17 (Elliott et al. 2008). Hence, by suppressing IL-17 *H. polygyrus* also has the potential to initiate a reduction in the pro-inflammatory cytokines IL-6 and TNF-α.

4.5.2. *No evidence of upregulated TH2 cytokine responses in co-infected wood mice*

Infection with *H. polygyrus* is known to modulate a variety of host immune responses. Specifically, the upregulation of Th2/immunoregulatory cytokine responses by *H. polygyrus* has been observed a number of cases (Bazzone et al. 2008, Reese et al. 2014). This upregulation of Th2 responses is thought to down-regulate Th1 responses, thus impairing the host's ability to deal with co-infecting microparasites. However, I was unable to demonstrate the upregulation of any Th2 cytokines in response to *H. polygyrus* infection; even so, we still see a marked reduction in Th1 cytokine expression in mice with current or previous *H. polygyrus* co-infections. Hence, it could be that up-regulated levels of other Th2 cytokines that were not measured in this study, such as IL-4 or IL-13, are responsible for the downregulation of Th1 cytokines shown. In particular, IL-4 has been shown to be upregulated in laboratory mice with *H. polygyrus* infections, and was particularly important in causing MHV-68 to re-active into an active infection (Reese et al. 2014). However, I was unable to measure IL-4 expression as I was unable to find IL-4 in the wood mouse genome. A second possibility is that any upregulation of Th2 cytokines were simply not visible in our data due to inadequate sampling time scales. For instance, Finney et al. (2007) found that *H. polygyrus* initiated a typical Th2 response early in infection, with high levels of Th2 cytokines 14-21 days post infection, subsequently becoming downmodulated after 21 days. Hence, we

may not see a substantial increase in Th2 responses from any mice that became infected with *H. polygyrus* over 21 days prior to capture.

I also found that in *H. polygyrus* co-infected mice expression of the Th17 pro-inflammatory cytokine TGF-β was downregulated compared to WMHV only infected mice. Conversely, *H. polygyrus* is known for inducing immunosuppression via T-reg cells and increased levels of TGF-β have been found in *H. polygyrus* infected mice (Finney et al. 2007, Bowron et al. 2020). Furthermore, TGF-β signalling has been found to be critical for the survival of *H. polygyrus* in the host, indeed *H. polygyrus* itself produces Excretory-Secretory (HES) antigens which contain a functional TGF-β mimic (Grainger et al. 2010) since TGF-β can downregulate the Th2 protective response needed for the host to clear helminth infections (Wilson et al. 2005). However, TGF-β is also known to be a driver of the highly pro-inflammatory Th17 cell subset which is very important in the anti-viral immune response (Eisenstein and Williams 2009).

4.5.3. *Cytokine gene expression in mice with lytic and latent WMHV*

By comparing cytokine gene expression among mice with WMHV infections I could determine if expression of any cytokines differed between mice with lytic or latent infections, and if those with *H. polygyrus* co-infections had reduced Th1 cytokine gene expression. Surprisingly, I also found no difference in expression of any cytokines between lytic or latent WMHV infections, and the only cytokine we found to be associated with *H. polygyrus* co-infection was *Tnf*-α expression, which shows that co-infected mice have reduced levels of *Tnf*-α expression compared to mice with only WMHV. Additionally, there was no increase in any of the Th2 cytokines (IL-10 and IL-5) in mice with *H. polygyrus* co-infections. It has previously been found that for *H. bakeri* infection to re-activate WMHV from latency there needs to be induction of the Th2 cytokine IL-4 and inhibition of Th1 responses, specifically INF-γ (Reese et al. 2014). Our data show that mice with *H. polygyrus* co-infections do not have suppressed INF-γ expression which is significantly different compared to mice with only WMHV infections; hence, this may explain why in the previous chapter we don't see mice with *H. polygyrus* co-infections being more likely to have lytic WMHV infections.

Conclusion

In conclusion, mice with current or previous *H. polygyrus* co-infections had reduced pro-inflammatory/Type 1 cytokine gene expression (*Tnf*-α, IL-6 and *Tgf*-β) compared to those with only WMHV infections. TGF- β has long been considered an immunoregulatory cytokine, however it has been found that TGF- β helps in the differentiation of the highly pro-inflammatory Th17 cells. Additionally, there was no evidence of Th2 cytokines being upregulated in response to *H. polygyrus* infection. However, there was no association between ivermectin treatment, WMHV infection and current or previous *H. polygyrus* FEC and expression of any cytokines.

5. Does *H. bakeri* co-infection increase susceptibility to active WMHV infections in wood mice?

5.1 Abstract

Most of our understanding of the immune response during co-infections has come from highly controlled laboratory settings involving laboratory mice kept in abnormally clean "specific pathogen free" (SPF) barrier facilities. These mice are often infected with unnatural host-parasite combinations, potentially causing different within-host interactions than if the natural host of the parasite were used. Hence, to fully understand the effect of co-infection on the host immune response it is necessary to moving more towards 'dirty' or wild animals with natural host-parasite combinations for these studies. Here I use controlled infection/co-infection experiments, with an outbred wild-derived, but now laboratory-reared colony of wood mice, to investigate the within-host interactions between the helminth *Heligmosomoides bakeri* (the laboratory species which is similar to that found to naturally infect wild wood mice) and WMHV co-infection under both high and low quality nutritional provisioning. Overall, these results show that H. *bakeri* co-infection reduces the ability of the host to control WMHV in mice on lower quality diets, but not in mice on higher quality diets. Additionally, I found that being co-infected with *H. bakeri* was associated with mice having a longer period of lytic infection in the lungs, however during the early stages of WMHV infection, co-infection seems to enable the to host to suppress lytic virus in the lungs to lower levels. Furthermore, there was some evidence of co-infected mice having higher type 2 cytokine (IL-5) expression, however I found the type 1 response to co-infection was very mixed.

5.2 Introduction

Most wild animal populations will be exposed to many different parasites and pathogens over the course of their lives, thus making co-infection with more than one species very common in nature (Petney and Andrews 1998, Cox 2001). Helminth infections are particularly important in co-infections since many parasitic worms can cause immunosuppression (Maizels et al. 2004, Maizels et al. 2012a, McSorley et al. 2013) and are incredibly common in most animal and human populations (Hotez et al. 2008). Consequently, lots of research has investigated the effects of helminth infection on the dynamics and transmission of co-infecting microparasites (viruses, bacteria etc). Several studies have shown that helminth co-infection can negatively impact the host's ability to respond to microparasitic co-infections (Edwards et al. 2005, Ayash-Rashkovsky et al. 2007, Reese et al. 2014). It has been hypothesised that these within-host interactions between helminths and pathogens are driven via the host immune system; specifically because helminth infection can skew the immune response towards a T-helper type 2 (Th2) phenotype, which along with the induction of immunoregulatory cytokines can downregulate the T-helper type 1 (Th1) anti-viral response (Mosmann and Sad 1996, Su et al. 2005, Anthony et al. 2007). (Mosmann and Sad 1996, Su et al. 2005, Anthony et al. 2007). However, it is also possible that within-host interactions between parasites and pathogens can occur through direct interactions, or indirectly through shared host resources (Pedersen and Fenton 2007). Given the ubiquity of helminth infection, it is crucial that we have a better understanding of how helminth infections alter the immune response to pathogens and how this impacts pathogen infection dynamics; as this will be important in the management of pathogenic infections of humans, livestock and wildlife.

During infection with microparasites such as viruses, a Th1 pro-inflammatory response is induced resulting in the production of cytokines Interleukin (IL) 2, IL-23, tumor necrosis factor (TNF)-α, and IFN- γ (Anthony et al. 2007, Alves et al. 2009, Moreau and Chauvin 2010). Additionally, Th-17 cells, a relatively newly discovered T-cell subset distinct from Th1 cells are also crucial for controlling microparasitic infections by secreting IL-17, IL-6 and TNF-α (Kolls and Lindén 2004, Langrish et al. 2005, Anthony et al. 2007). These cytokines produced during a Th1 immune response can activate $CD8^+$ cytotoxic T cells which kill virus-

infected cells, induce neutralising IgG antibodies against virus particles and cause inflammation all these mechanisms are crucial for reducing viral loads and associated pathology. In contrast, during infection with macroparasites such as helminths, a Th2 response is induced resulting in the release of cytokines IL-4, IL-5, IL-9, IL-10 and IL-13 which aid in mounting an effective immune response (i.e. clearing infection/reducing parasite burdens and reducing disease caused by infection) (Anthony et al. 2007). These Th2 cytokines enable helminth expulsion from the gut by inducing eosinophil differentiation, activating B cells (Chan et al. 1992, Anthony et al. 2007), and inducing alternatively activated macrophages (Weng et al. 2007). Some helminths can actively supress this Th2 pathway to improve their survival, and ability to infect and transmit offspring to new host. For example, helminths are strong inducers of regulatory T-cells (Treg) which secrete TGF-β and IL-10 which can downregulate both Th1 and Th2 responses (Su et al. 2005, Hang et al. 2019). One of the most well-studied laboratory models of immunomodulatory helminth species is the murine intestinal nematode *Heligmosomoides bakeri* (Behnke and Harris 2010), a model of human hookworm infection (Monroy and Enriquez 1992, Maizels et al. 2012a). *H. bakeri* is known for modulating a wide variety of host immune responses, such as reducing antibody responses to influenza A infections (Chowaniec et al. 1972), exacerbating infection with *plasmodia spp* and increasing host mortality (Helmby 2009, Tetsutani et al. 2009). Additionally, *H. polygyrus* co-infection with *Schistosoma mansoni* was found to reduce schistosome egg-induced hepatic immunopathology which was associated with decreased levels of IL-17, INF-γ, TNF-a, IL-23 and IL-6, and increased levels of IL-4, IL-5, IL-10 and TGF- β (Bazzone et al. 2008), and re-activated murine gamma-herpes virus (MHV-68) from latency via induced IL-4 (Reese et al. 2014). Hence, it is clear that the immunomodulatory properties of *H. bakeri* leads to strong within-host interactions with microparasites; and as such it has become an important model organism in laboratory co-infection studies.

5.2.1. *The effect of nutrition on the host immune response*

In addition to being impacted by within-host parasite interactions, a host's ability to mount an effective immune response can also be affected by its nutritional status. Hence, it is unsurprising that animals on lower quality diets have been found to be less able to cope with parasitic infections (i.e. they are less able to effectively clear infections and may have more sever disease outcomes) (Cypher and Frost 1999, Coop and Kyriazakis 2001, Ezenwa

2004). For example, kit foxes in California from residential areas, where anthropogenic food sources are available, were found to be heavier, in better condition and consequently had better stronger immune responses, than animals in a reserve, with less readily-available food sources (Cypher and Frost 1999). Also, Ezenwa (2004) found that wild bovids that were unable to maintain adequate nutrition during drought years, were less able to cope with gastrointestinal parasites, with reduced protein intake during dry periods being associated with reduced resistance to, and ability to recover from infection. Furthermore, energy deficits in laboratory mice have been shown to simultaneously suppress both Th1 (INF-γ) and Th2 (IL-4 and IL-5) responses and their effector functions (Koski et al. 1999). Examples of which include IL-4 being suppressed in the spleens of zinc deficient mice (Scott and Koski 2000), and protein malnutrition being associated with increased survival of *H. polygyrus*, reduced levels the Th2 cytokine IL-4, but increased levels of the Th1 cytokine INF-g (Ing et al. 2000). It is becoming clear that poor nutrition can have consequences for the host immune system, potentially reducing Th2 cytokine responses and upregulating Th1 pro-inflammatory responses.

5.2.2. *Comparing lab and wild animal models of infection*

Most studies on the immune response during co-infections have involved laboratory mice (*Mus musculus*) kept in clean, controlled "specific pathogen free" (SPF) barrier facilities (Babayan et al. 2011, Beura et al. 2016a). As a consequence, most laboratory co-infection studies have not investigated the impact of poor nutrition, since all animals are maintained on the same, high quality diets. These highly controlled experiments have eliminated as much experimental variation as possible; even so, the translation of findings from preclinical animal models to clinical efficacy and safety in humans has been notoriously unreliable, meaning that the majority of clinical trials do not result in approved human treatments (Hay et al. 2014, Harrison 2016, Pound and Ritskes-Hoitinga 2018). This is unsurprising since laboratory mice have limited genetic diversity, are not constantly exposed to different parasites as are wild animals (Pedersen and Babayan 2011, Maizels and Nussey 2013, Cadwell 2015), and have immune systems comparable to those of neonatal humans rather than of adult humans (Beura et al. 2016a). For example, the cellular immune systems of wild mice were found to be in a highly activated state compared to those of in-bred laboratory mice, despite this cells from wild mice had lower *in vitro* cytokine responses to pathogen-

associated ligands compared to laboratory mice, potentially indicating the importance of maintaining immune homeostasis in the wild (Abolins et al. 2011). Furthermore, 'dirty' pet store and wild mice have been found to have higher numbers of differentiated memory T cells in lymphoid and non-lymphoid tissue, which more closely matches the immune systems of adult humans than genetically-deficient laboratory mice raised in SPF conditions (Beura et al. 2016a). Another reason why results from laboratory mouse models may not be reflected in natural populations is that many of these studies use unnatural host-parasite combinations, potentially causing different with-host interactions than if the natural host of the parasite were used. Thus, this suggests that to more closely model the immune response of humans and wild animals, we should move away from using inbred laboratory mice infected with unnatural parasite combinations and move more towards outbred or 'dirty' animal models who are the natural host of the parasites, and may be subject to imperfect conditions (e.g. co-infection, lower quality diets).

5.2.3. *The wood mouse system as a natural model of co-infection*

The wood mouse (*Apodemus sylvaticus*) is a common wild mouse species living in woodlands in the UK and Europe (Knowles et al. 2013). Wood mice are commonly infected with both wood mouse herpes virus (WMHV) and the nematode *H. polygyrus* (sister species of the *H. bakeri* lab model), both of which are closely related to current laboratory mouse models of infection. Wood mouse herpes virus is a gamma-herpesvirus that is commonly found in mice and other murid rodents (Telfer et al. 2007), and its closely related laboratory species, MHV-68, has been well studied in laboratory mice. MHV-68 infection in laboratory mice usually starts with a lytic phase of infection (most viral genes are expressed, and the virus can replicate) in the lungs before moving to the spleen (Sunil-Chandra et al. 1992a, Flaño et al. 2005). Lytic virus replication in the lungs can be detected from 1 day post infection (dpi), with peak titres at 7 dpi and is usually cleared from the lungs by 10 dpi (Flaño et al. 2005). Furthermore, latent (non-replicating) virus has been found in the lungs of mice from as early as 3 dpi with levels of latent virus remaining high before declining to low stable levels by 30 dpi (Flaño et al. 2005). From the lungs the virus then spreads to the spleen, with infectious virus detectable at low levels from 3 dpi, with latent virus detectable from 6 dpi and peak latent infection is reached around 14 dpi before declining to low stable levels by day 90 days post infection (Sunil-Chandra et al. 1992a, Flaño et al. 2005). WMHV

then establishes a latent and lifelong infection, mainly in splenic B cells, but also in macrophages and dendritic cells (Flaño et al. 2000, Nash et al. 2001, Flaño et al. 2005) and lung epithelial cells (Stewart et al. 1998). The lifecycle of rodent gammaherpes viruses was investigated in laboratory mice, however Hughes et al. (2010b) studied the progression of lytic MHV-68 infection in the lungs and latent infection in the spleens of wood mice, and found that progression of MHV-68 in wood mice is similar to that in laboratory mice. Noticeably, the main differences were that infectious virus in the lungs could not be detected until day 7 and there were lower levels of latent virus in the spleen after 20 days post infection (Hughes et al. 2010b).

During latent infection very few viral genes are expressed, however open reading frame 73 (*ORF*73) is one of the few expressed throughout latency and *ORF*73 has been shown to be critical for establishing and maintaining viral infection in latently infected cells (Fowler et al. 2003, Marques et al. 2003). This latent virus then has the molecular ability to reactivate into a lytic infection (Wu et al. 2000, Wu et al. 2001), controlled by the gene *rta* (replication and transcription activator), primarily encoded by open reading frame 50 (*ORF*50) (Liu et al. 2000, Wu et al. 2000, Wu et al. 2001). As mentioned previously, *H. polygyrus* is known to induce a highly polarized Th2 immune response in the host, and has the ability to suppress the host immune system via Treg cells (Maizels et al. 2012a), allowing for potential immune-modulated interactions between WMHV and *H. polygyrus*. Taken together, this makes wood mice an excellent and tractable model to assess the interactions between helminths and viral pathogens in their natural host under a range of natural conditions (e.g. poor nutrition).

5.2.4. *Hypotheses*

In this study used controlled infection/co-infection experiments, with an outbred wild-derived, but now laboratory reared colony of wood mice, to investigate the within-host interactions between *H. bakeri* (the laboratory strain of the species found to naturally infect wild wood mice) and WMHV co-infection under both high and low quality nutritional provisioning. Since *H. bakeri* is known to downregulate the Th1 immune response which would therefore increase host susceptibility to viral infection, I predict that mice infected with *H. bakerii* would have higher viral titres and a longer period of lytic infection; and that

this effect may be compounded in mice on a poorer quality diet. Next, I aimed to investigate the mechanistic basis for these within-host interactions, by exploring whether *H. bakeri* infection alters Th1 cytokine expression which are important for controlling WMHV infections, and if this relationship is affected by nutrition. Based on previous work, I expected that *H. bakeri* infection will lead to an increase in expression of type 2 cytokines (IL-5 and IL-10), and consequently a reduction in expression of type 1 cytokines (*Tnf*-α, *Inf*-γ, IL-6, and *Tgf*-β) compared to mice only infected with WMHV. Additionally, I hypothesised that WMHV-only infected mice have higher pro-inflammatory Th1 cytokine expression compared to co-infected mice. Hence, they could show higher expression levels of anti-inflammatory cytokines, specifically IL-10 as this is an immunoregulatory and is produced in response to inflammation. It is also expected that mice on higher quality diets would be more able to mount an effective immune response, leading to a reduction in worm burdens, and lower viral titres of WMHV in both single and co-infected mice.

This chapter aims to answer the following questions;

1) Does co-infection with *H. bakeri* affect *ORF*50 or *ORF*73 expression in the spleen?
2) Does co-infection with *H. bakeri* affect *ORF*50 or *ORF*73 expression in the lungs?
3) Does *H. bakeri* co-infection downregulate the expression of Th1 cytokines in the spleen?
4) Does *H. bakeri* co-infection downregulate the expression of cytokines aimed at WMHV infection in the lungs?

5.3 Methods

5.3.1. Source of virus stocks and Heligmosomoides bakeri

Wood Mouse Herpes virus (WMHV) which was isolated from wild wood mice in Cheshire during 2002 and cultured from homogenized lung tissue was provided by Prof. James Stewart (Hughes et al. 2010a). It was recently tested to ensure that it was not contaminated with any other potential pathogens (IMPACT testing).

The laboratory maintained helminth species *H. bakeri* provided by Dr. Amy Buck at the University of Edinburgh was used in this experiment; this species is closely related to the species found to infect wood mice in their natural habitat (Behnke and Harris 2010).

5.3.2. *Wood mouse colony*

Wood mice (*Apodemus sylvaticus*) were maintained under standard laboratory conditions at the University of Edinburgh animal husbandry facilities. The wood mouse colony was originally derived from wild caught animals collected from a woodland in the Wirral, UK and have since been outbred to maintain as much genetic variability as possible. Wood mice were between 10-20 weeks old and a near equal ratio of males and females. All wood mice were co-housed in single sex groups of 6 animals and were individually identified by ear notching.

5.3.3. *Experimental design*

Our experimental design was composed of three infection groups, all of which were challenged with WMHV infection (see details below): 1) *H. bakerii* prior infection, 2) *H. bakerii* chronic infection, and 3) WMHV only (Figure 5.1). Each group had 20 age and sex-matched wood mice, half of which were fed on a high-quality Transbreed diet, and the other half were fed on a standard-quality diet resulting in 6 experimental groups in total (3 infection regimes x 2 diets). Fourteen days before the start of the experiment, 10 mice were randomly assigned to each of the 6 experimental groups, plus 4 mice randomly assigned to cages as transmission controls (sentinel mice to test for any within-cage transmission of either WMHV or *H. bakeri*). This led to a total of 64 wood mice, all of which were co-housed and fed their experimental diets for 2 weeks prior to any inoculations. The time schedule and details for each experimental group were as follows:

Day -14: Mice were grouped, co-housed and fed with their experimental diets.

Day 0: Mice from group 1 (*H. bakeri* prior infection) and group 2 (*H. bakeri* chronic infection) received 150 *H. bakerii* L3 infective stage larvae in 150µl of water via oral gavage. Group 3 (WMHV only) and transmission controls, received an equivalent (150µl) dose of water via oral gavage.

Day 14-16: All wood mice were transferred to clean cages for 4 hours to collect faecal samples to measure *H. bakeri* infection and egg shedding.

Day 21: All wood mice in group 2 (*H. bakeri* chronic) received a second dose of 150 *H. bakerii* L3 infective stage larvae in 150µl of water via oral gavage in an effort to maintain infection levels and generate chronic *H. bakeri* infections (Clerc et al. 2019b). Wood mice in group 1 (*H. bakeri* prior), received a weight adjusted dose (2µl/g) of a set of two anthelmintic drugs: Ivermectin and Pyrantel (9.4mg/kg Ivermectin and 100mg/kg Pyrantel) via oral gavage which have been shown to eliminate *H. bakeri (Clerc et al. 2019a)*. The WMHV only group and transmission controls received 150µl of water via oral gavage.

Day 28: All wood mice (except transmission controls) were intranasally inoculated with $4x10^5$ plaque forming units of WMHV (in 40µl PBS) under light anaesthetic following Hughes et al. (2010b).

Day 35, 42 & 72: Two wood mice from each of the 6 experimental groups were culled and tissues harvested on each day (35, 42 and 72; see below), all transmission controls were culled at day 72. These time points correspond to 7 days (peak lytic infection in the lungs), 14 days (peak latent infection in the spleen) and 35 days (persistent infection established in the spleen and lungs) post WMHV infection.

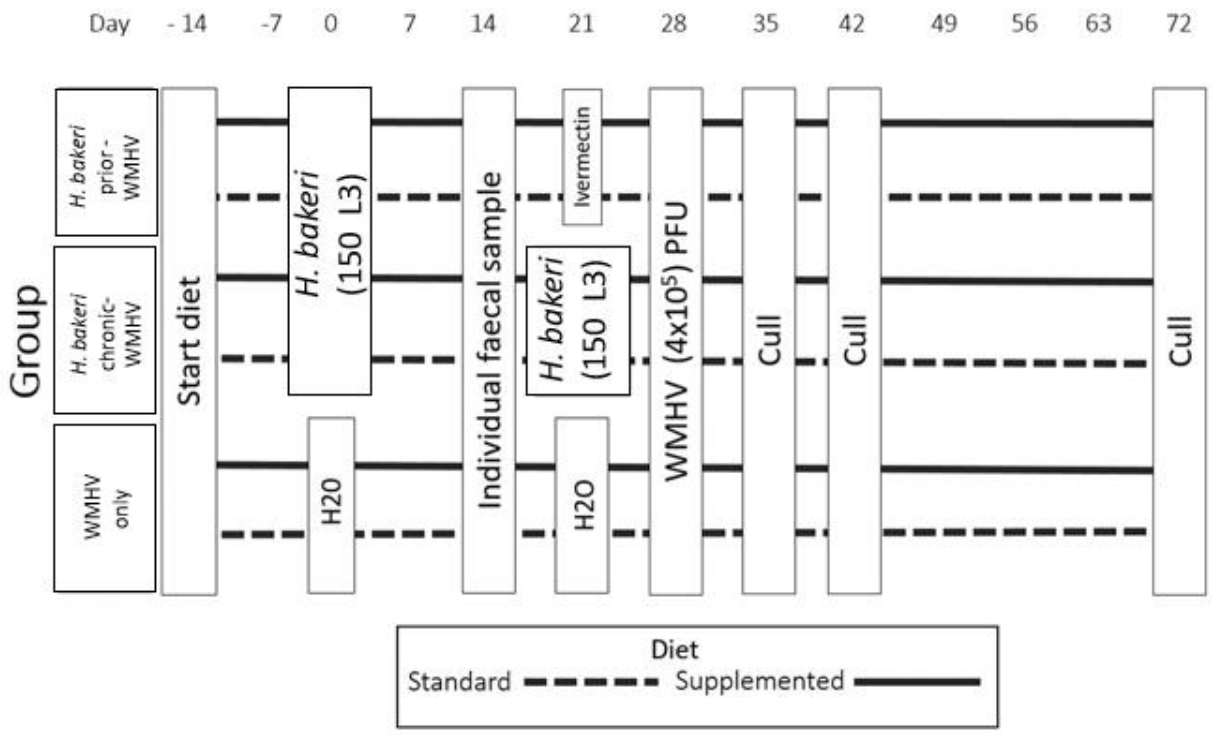

Figure 5.1. Experimental design. Wood mice were split into 3 groups (WMHV-only, *H. bakeri* chronic, *H. bakeri* prior) and fed their experimental diets for 14 days before being given their dose of *H. bakeri* larvae or water. On day 21 mice in the *H. bakeri* prior group were given a weight adjusted does of ivermectin, those in the H. bakeri chronic group were given a second dose of *H. bakeri* larvae and WMHV-only group were given water. On day 28 all mice were intranasally inoculated with $4x10^5$ PFU WMHV. Mice were then culled on days 35, 42 and 72.

5.3.4. *RNA extraction and cDNA synthesis*

I extracted RNA from both lung and spleen tissue that had been sorted in RNAlater at -80°C, using RNeasy® PowerLyzer® Tissue & cells kit (Qiagen) according to instructions, including on column DNase 1 treatment. Next, I measured optical density of each RNA sample to determine RNA concentration before running an agarose gel to ensure there was no DNA contamination. cDNA was reverse transcribed from RNA by first heating 2µg total RNA in the presence of random primers, dNTP's and RNase free water (total volume of 13µl) to 65 degrees, before snap cooling on ice and adding first strand synthesis buffer plus 0.1M DTT, then heating to 42°C before adding 1µl RTase (SuperScript II) and incubating at room temperature. The mixture was then incubated at 42°C for 2 hours, then inactivated at 70°C. Samples were stored at -20°C until needed.

5.3.5. *Real-time quantitative RT-PCR analysis*

Quantification of viral *ORF*50 (as a measure of lytic WMHV infection) and *ORF*73 (as a measure of both active and latent infection) gene expression in spleen and lung samples was performed using a LightCycler480 for method see section 4.3. Additionally, a 100bp portion of the WMHV gene *ORF*50 was amplified using the forward primer 5'-CATCCGAGGACGCGTTCATA-3' and reverse 5'-GTGGAACACACATCACTGTC-3' (acc. no. GQ169129.1).

For details on RT-qPCR for cytokine gene expression refer to Methods section 4.2.1.

All RT-qPCR reactions were carried out in duplicates and all samples used in the analysis had duplicate ct values that were within 0.5 sd of each other.

5.3.6. *Statistical analyses*

All statistical analysis was performed in R, v. 3.1.5 (R development core team 2011).

For all analyses the two helminth infection groups (prior *H. bakeri* and chronic *H. bakeri)* were combined into one 'co-infected' variable since preliminary analyses suggested that having them separate did not provide any additional insight in either the spleen (see Supplementary Material; Figure 1: Table 1) or the lungs (see Supplementary Material; Figure 2: Table 2), so they were combined for simplicity. Since chronic *H. bakeri* and prior *H. bakeri* were combined in the first analysis (the effect of infection group and diet on WMHV gene expression) they were also combined for all subsequent cytokine analyses.

Worm FEC was not used in the analyse since very few mice were shedding *H. bakeri* eggs (26% of mice were shedding eggs), hence there were some groups in which all mice were not shedding eggs making the analysis meaningless.

Analyses were conducted to address the following questions;

1) Does co-infection with *H. bakeri* affect *ORF*50 or *ORF*73 expression in the spleen?

To determine if WMHV infection (detectable *ORF*73 expression; represented as factor: yes or no) was associated with infection group (factor: WMHV only, *H. bakeri*-co-infected) or diet (factor: standard quality, high quality) I ran a binomial GLM:

WMHV infection ~ time point * (diet *Infection group + Sex).

Next, I ran a second binomial GLM to determine if, among those with detectable infections (detectable *ORF73* expression), having a lytic WMHV infection (detectable *ORF*50 expression) was associated with infection group or diet:

*ORF*50 ~ time point * (diet *Infection group + Sex).

In all models I controlled for the following variables: sex (factor: female, male) and time point (days since WMHV infection; factor: 7, 14 and 35). Interactions were also included to test for time specific sex effects (days since infection* sex), and a three-way interaction to test for time and diet specific infection group effects (days since infection *diet* infection group). In all cases, full models were created for each response variable above, and then models were simplified by backwards elimination of non-significant terms initially with a cut off at ($p<0.07$), starting with interactions, to obtain a minimal model with all terms $p<0.05$.

To determine if *H. bakeri* co-infection was associated with higher levels of *ORF*50 (WMHV lytic infection) or *ORF*73 (WMHV infection) in the spleen; I only included wood mice with detectable levels of *ORF*50 or *ORF*73 expression (>0). I modelled this using two gaussian GLM's to determine if expression of (i) *ORF*50 or (ii) *ORF*73 (continuous, log transformed) was associated with infection group (factor: WMHV only, co-infected) or diet (factor: standard quality, high quality):

(i) *ORF*50 quantity spleen ~ time point * (diet *Infection group + Sex)

(ii) *ORF*73 quantity spleen ~ time point * (diet *Infection group + Sex)

In each model, as described above I included covariates, relevant interactions, and model simplification.

2) <u>Does co-infection with *H. bakeri* affect *ORF*50 or *ORF*73 expression in the lungs?</u>

To determine if *H. bakeri* co-infection was associated with (i) detectable WMHV infection (detectable *ORF73* expression), or (ii) lytic WMHV infection (detectable *ORF50* expression) in the lungs, I conducted a similar set of analyses as above, but using data from lung samples:

(i) *ORF*50 quantity lungs ~ time point * (diet *Infection group + Sex)

(ii) *ORF*73 quantity lungs ~ time point * (diet *Infection group + Sex)

3) Does *H. bakeri* co-infection downregulate the expression of Th1 cytokines in the spleen?

To determine if *H. bakeri* infection affects cytokine gene expression, I modelled the level of expression for each cytokine separately (Th1: TNF-α, INF-γ , TGF-β, and IL-6; Th2: IL-5, IL-10) at each time point (days since WMHV infection) as the response variables. All models were Gaussian GLM's with relative expression of each cytokine (square root transformed), as the response variable, and infection group (factor: WMHV only, co-infected) and diet (factor: standard quality, high quality) as the main explanatory variables of interest:

Cytokine expression in spleen ~ diet *Infection group * Sex

All models controlled for sex (factor: female, male), and included a three-way interaction to test for sex and diet specific co-infection effects (sex*diet* infection group). All models were simplified as above. Adjusted P-values for terms in the minimal model (q-values) were calculated based on the 'Graphically Sharpened' false discovery rate method (Pike 2011).

4) Does *H. bakeri* co-infection downregulate the expression of cytokines aimed at WMHV infection in the lungs?

To determine if *H. bakeri* infection affects cytokine gene expression in the lungs, I conducted a similar set of analyses as above, but using cytokine levels of gene expression from lung samples:

Cytokine expression in lungs ~ diet *Infection group * Sex.

Adjusted P-values for terms in the minimal model (q-values) were calculated based on the 'Graphically Sharpened' false discovery rate method (Pike 2011).

5.4 Results

5.4.1 *Does co-infection with H. bakeri affect ORF50 or ORF73 expression in the spleen?*

There was a diet-specific infection group effect (diet* infection group interaction) (x^2_1=5.589, p=0.018; Table 5.1a, Figure 5.2a) on the probability of having a detectable WMHV infection (*ORF*73 expression) in the spleens. *H. bakeri* co-infection was associated with increased probability of mice having a detectable WMHV infection at days 7 and 35 post infection when on lower-quality standard diets, compared to WMHV-only mice on the same diet. All mice across both diets had detectable infections in their spleens at day 14 post infection and there was no effect of co-infection among mice on high-quality diets. Additionally, there was a significant main effect of sex on the probability of having detectable infection across both diets and infection groups (x^2_1=5.473, p=0.019), suggesting that females were less able than males to suppress *ORF*73 below detectable levels in the spleen (Figure 5.2a). There was also a significant time point*diet interaction (x^2_2=6.720, p=0.018) (Table 5.1a, Figure 5.2a). However, in mice that had detectable *ORF*73 expression there was no association between co-infection and *ORF*73 quantity (x^2_1=0.087, p=0.768; Table 5.1b); the only variable that remained in the model was time point (x^2_2=32.933, p<0.001), showing that expression levels were lowest 7 days post infection, which increased at 14 days post infection, before decreasing again by day 35 (Table 5.1b, Figure 5.2b).

In contrast, helminth co-infection was not associated with the probability of having an active WMHV infection (detectable *ORF*50 expression) in the spleen (x^2_1=0.01, p=0.91; Table 5.1c). Again, the probability of having detectable *ORF*50 expression varied significantly across time points (x^2_2=18.40, p<0.0001); around 35% of mice had detectable ORF50 expression 7 days post-infection, rising to all mice having detectable expression at 14 days post infection, declining to about 75% of mice at 35 days post-infection (Table 5.1c, Figure 5.2c). Again, infection group was not associated with expression levels of *ORF*50 (x^2_1=1.452, p=0.228; Table 5.1b). However, time point (x^2_2=31.907, p<0.001) and sex (x^2_1=4.029, p=0.0447) remained in the model, with the highest *ORF*50 expression 14 days after WMHV infection, and females having higher expression of *ORF*50 than males over all time points (Table 5.1d, Figure 5.2d).

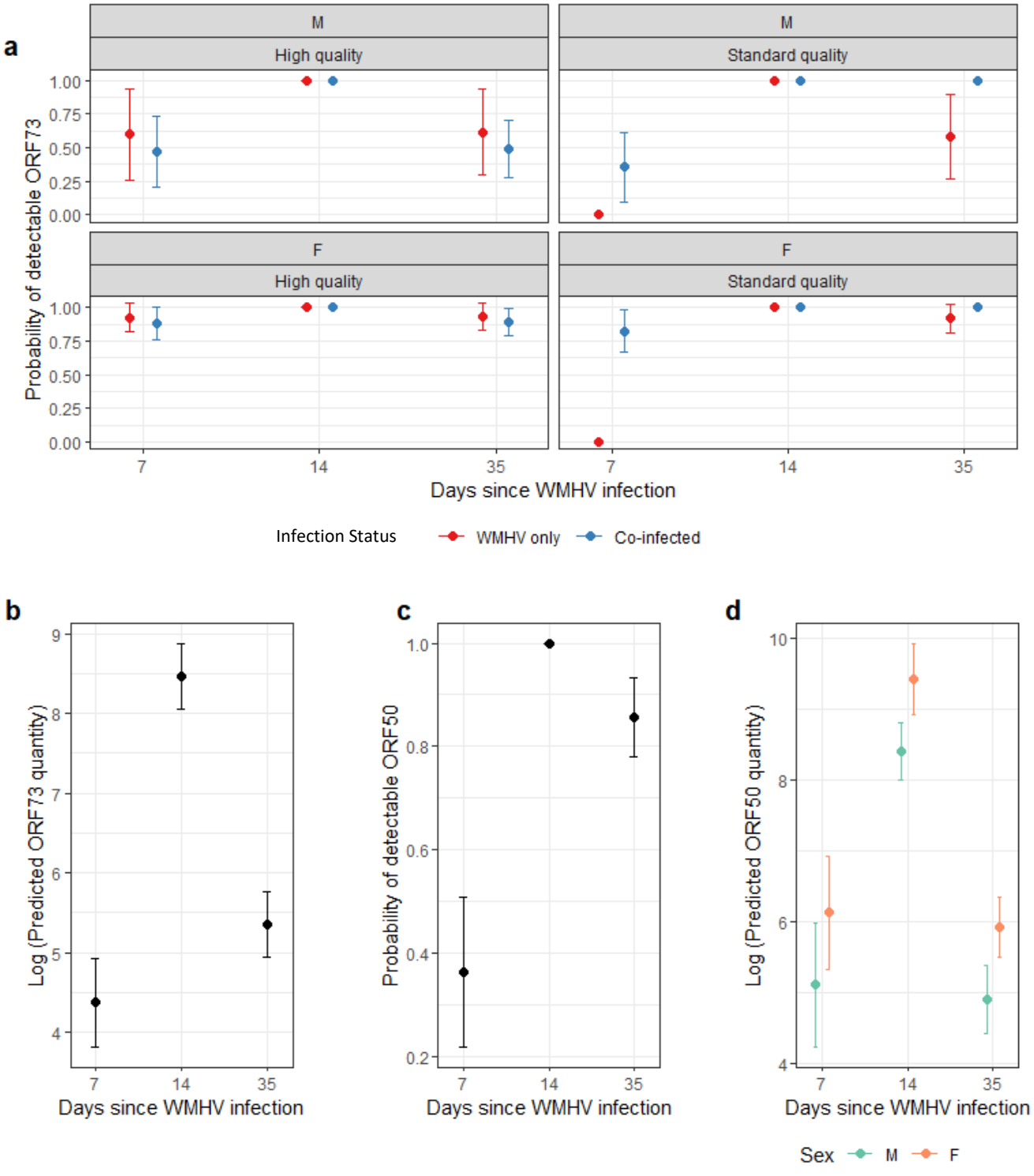

Figure 5.2. Association between a) probability having a detectable WMHV infection (detectable *ORF*73), diet, sex and infection group, b) *ORF*73 quantity and days since WMHV infection, c) probability of lytic infection (detectable *ORF*50 expression) and days since WMHV infection, and d) *ORF*50 quantity, sex and days since WMHV infection in laboratory bread wood mice; (n=60). Co-infection with *H. bakeri* is associated with reduced ability to suppress *ORF*73 below detectable levels in mice on standard diets.

Table 5.1. Factors affecting WMHV gene expression levels in the spleens of laboratory bred wood mice infected with WMHV. Models were simplified by backwards stepwise elimination. For variables not in the minimal model, statistics are those at the point they left the model. The variable 'time' refers to the number of days since WMHV infection.

		A. *ORF*73 p/a (AIC: 52.86)		**B.** *ORF*73 quantity (AIC:208.59)	
Variable	df	X^2	p	X^2	p
Time*Diet* infection group	1	0.0000	1.0000	1.6971	0.1927
Time* infection group	2	2.7203	0.1871	0.7083	0.7018
Time*Diet	2	6.7201	0.0181 ¥	0.9464	0.6230
Time*Sex	2	2.8237	0.2437	1.6337	0.2012
Diet* infection group	1	5.5894	0.0181 ¥	0.0606	0.8055
Diet	1	-	-	0.6510	0.4197
Sex	1	5.4734	0.0193 ¥	0.1165	0.7328
infection group	1	-	-	0.0870	0.7680
Time	2	-	-	32.933	<0.0001 ¥
		C. *ORF*50 p/a (AIC: 57.37)		**D.** *ORF*50 quantity (AIC: 167.12)	
Variable	df	X^2	p	X^2	p
Time*Diet* infection group	1	3.7798	0.1511	1.6186	0.2033
Time* infection group	2	0.4833	0.7853	0.3108	0.5772
Time*Diet	2	1.7122	0.4248	0.5130	0.7738
Time*Sex	2	0.0594	0.9707	0.1398	0.5656
Diet* infection group	1	0.1786	0.6726	0.0667	0.7962
Diet	1	0.9102	0.3401	0.1540	0.2828
Sex	1	1.1368	0.2863	4.0290	0.0447 ¥
infection group	1	0.0138	0.9063	1.4520	0.2283
Time	2	18.395	0.0001 ¥	31.907	<0.0001 ¥
¥ Variables in the minimal model (P < 0.05)					

5.4.2 *Does co-infection with H. bakeri affect ORF50 or ORF73 expression in the lungs?*

Similar to data from the spleen, there was a significant diet specific effect of infection group on the probability of having a detectable WMHV infection (detectable *ORF*73 expression) (x^2_1=6.031, p=0.014; Table 4a, Figure 5.3a). Once again, co-infected animals on lower-quality diets were more likely to have detectable WMHV infections at days 7 and 35 post infection compared to those with only-WMHV infections on the same diet. Again, all mice across both diets had detectable infections in the lungs at day 14 post infection and there was no association between *H. bakeri* co-infection and the probability of having detectable infections in those on high quality diets. Additionally, there was a significant overall main effect of sex (x^2_1=20.56, p<0.001), and time point (x^2_2=23.60, p<0.001) on the probability of

having a detectable infection (Table 5.2a, Figure 5.3a). In particular, as with the analyses from the spleens, females tended to have higher probabilities overall of having a detectable WMHV infection. Additionally, among those with detectable infections infection group was not associated with *ORF73* quantity (x^2_1= 0.040, p=0.842), only time point remained in the model (x^2_2=12.044, p=0.002) (Table 5.2b, Figure 5.3b).

In contrast to data from the spleen, in the lungs there was a significant association between infection group (x^2_1=5.66, p=0.02) and time point (x^2_2=28.57, p<0.0001) and the probability of having detectable *ORF*50 expression (Table 5.2c, Figure 5.3c). At day 35 co-infected mice were more likely to have detectable *ORF*50 expression compared to those with only WMHV infections. However, in those with detectable *ORF*50, *H. bakeri* co-infection was associated with lower levels of *ORF*50 expression (x^2_1=7.867, p=0.005). Again, time point remained in the model, showing that levels of *ORF*50 expression varied with days post WMHV infection (x^2_2=18.569, p<0.001) (Table 5.2d, Figure 5.3d).

Table 5.2. Factors affecting WMHV gene expression levels in the lungs of laboratory bred wood mice infected with WMHV. Models were simplified by backwards stepwise elimination. For variables not in the minimal model, statistics are those at the point they left the model. The variable 'time' refers to the number of days since WMHV infection.

		A. *ORF*73 p/a (AIC: 38.32)		**B.** *ORF*73 quantity (AIC: 157.56)	
Variable	df	X^2	p	X^2	p
Time*Diet* infection group	1	0.0000	1.0000	1.3563	0.0575
Time* infection group	2	4.8917	0.0867	4.7232	0.0943
Time*Diet	2	0.0000	1.0000	3.1497	0.2070
Time*Sex	2	4.4987	0.1055	2.0039	0.3672
Diet* infection group	1	6.0309	0.0141 ¥	1.7421	0.1869
Diet	1	-	-	0.0773	0.7810
Sex	1	20.555	<0.0001 ¥	2.0923	0.1480
infection group	1	-	-	0.0400	0.8415
Time	2	23.597	<0.0001 ¥	12.044	0.0024 ¥
		C. *ORF*50 p/a (AIC: 42.82)		**D.** *ORF*50 quantity (AIC: 130.36)	
Variable	df	X^2	p	X^2	p
Time*Diet* infection group	1	0.0000	1.0000	0.2286	0.6326
Time* infection group	2	0.0000	1.0000	0.8586	0.3541
Time*Diet	2	0.0000	1.0000	2.3050	0.3158
Time*Sex	2	0.0000	1.0000	0.6654	0.7170
Diet* infection group	1	0.0000	1.0000	1.1328	0.2872
Diet	1	0.6438	0.4223	0.2693	0.6038
Sex	1	0.1165	0.7328	0.4508	0.5020
infection group	1	5.6612	0.0173 ¥	7.8666	0.0050 ¥
Time	2	28.568	<0.0001 ¥	18.569	<0.0001 ¥
¥ Variables in the minimal model ($P < 0.05$)					

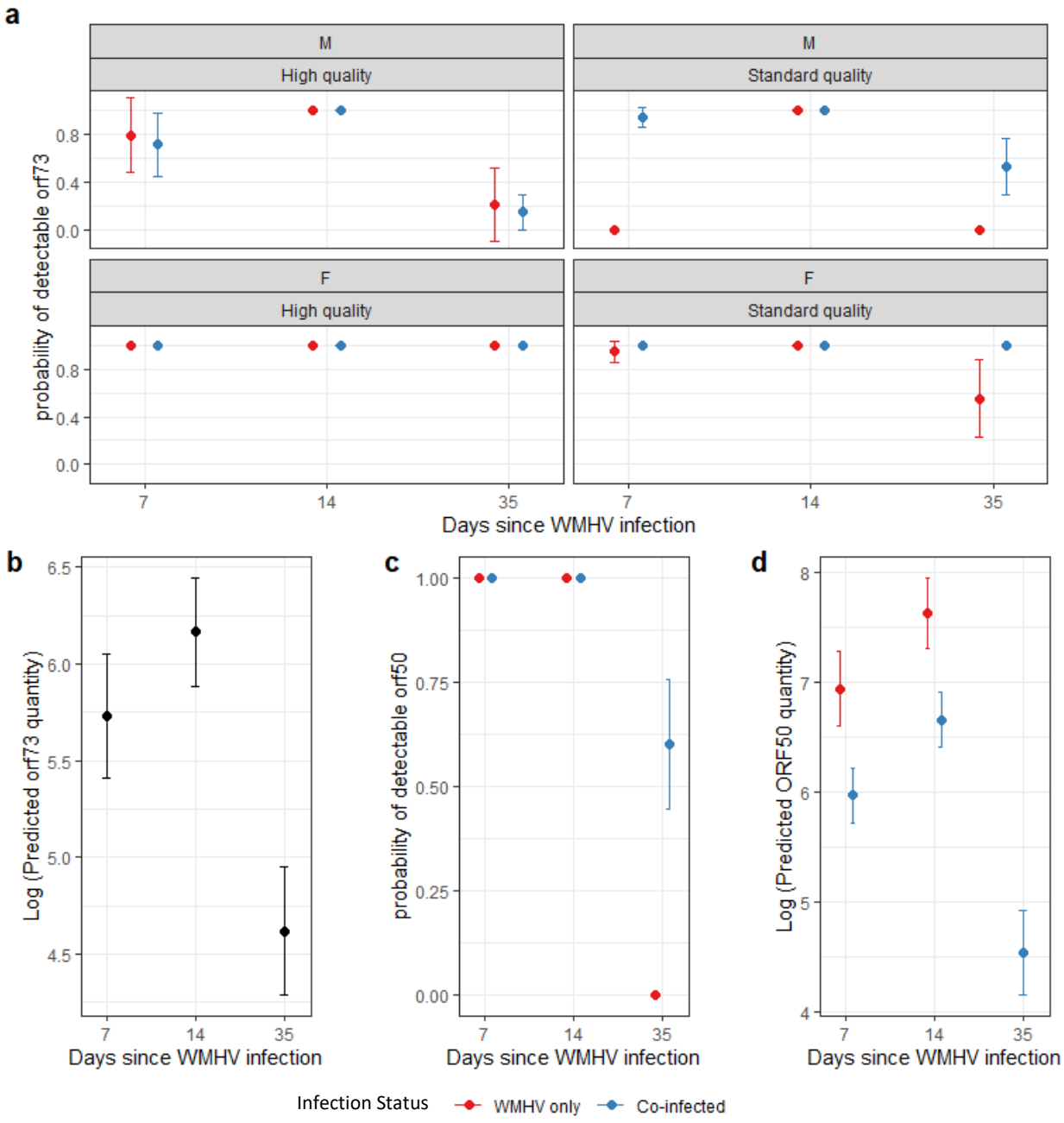

Figure 5.3. Association between **a)** probability of detectable WMHV infection (detectable *ORF*73 expression), sex, diet, infection group and days since WMHV infection, **b)** *ORF*73 quantity and days since WMHV infection, **c)** probability of detectable *ORF*50, infection group and days since WMHV infection and **d)** *ORF*50 quantity, infection group and days since WMHV infection in the lungs of laboratory bred wood mice; (n=60). *H. bakeri* co-infections were associated with reduced ability to suppress *ORF*73 (in mice on standard quality diets) and *ORF*50 (at day 35 post WMHV infection) expression below detectable levels. Additionaly, in those with detetable *ORF*50, co-infected mice at days 7 and 14 had higher *ORF*50 quantites compared to WMHV-only mice, there were no WMHV-only mice with detectable *ORF*50 expression at day 35.

5.4.3 Does *H. bakeri* co-infection downregulate the expression of cytokines aimed at WMHV infection in the spleen?

Type 1 cytokines in the spleen

Inf-γ gene expression was significantly affected by an infection group *diet*sex interaction at day 14 (x^2_1=4.08, q=0.049; Table 5.3b; Figure 5.4a) and an infection group *sex (x^2_1=6.12, q=0.032) and diet*sex (x^2_1=3.87, q=0.049) interaction at day 35 (Table 5.3c; Figure 5b). At day 14 there was an effect of co-infection in males on lower quality standard quality diets; they had lower *Inf*-γ expression compared to WMHV-only males on the same diet, but there was no effect of co-infection on *Inf*-γ expression for high quality males or females on both diets (Figure 5.4a). Additionally, in males with WMHV-only infections and co-infected females, lower quality diets increased *Inf* -γ expression compared to those on higher quality diets. At day 35, co-infected females on both diets had higher *Inf* -γ expression than WMHV-only mice (Figure 5b). In contrast, at day 7 there was no association between *Inf* -γ expression and infection group (x^2_1=1.08, p=0.18; Table 5.3a).

Tnf-α gene expression was significantly associated with infection group in animals on standard quality diets at day 14 (x^2_1=13.04, q=0.004; Table 5.3e, Figure 5.4c). Co-infected animals on standard quality diets had reduced *Tnf*-α gene expression compared to WMHV-only infected animals. Additionally, across both co-infected and WMHV-only infected those on standard quality diets had increased *Tnf*-α expression compared to those on high quality diets, and there was no association between infection group and *Tnf*-α expression in animals on high quality diets. Furthermore, there was no association between *Tnf*-α gene expression and infection group at day 7 (x^2_1=0.09, p=0.76; Table 5.3d) or day 35 (x^2_1=0.03, p=0.86; Table 5.3f); however, there was a significant effect of diet on day 7 (x^2_1=5.11, q=0.033; Table 5.3d). Animals on standard quality diets had higher *Tnf*-α gene expression compared to those on high quality diets.

Finally, *Tgf*-β expression had a significant diet*infection group infection group interaction (x^2_1=5.21, 0.033), sex*infection group interaction (x^2_1=6.02, 0.032), and a diet*sex interaction (x^2_1=4.93, q=0.033) at day 14 (Table 5.3h, Figure 5.4d). Co-infected males on both diets had lower TGF-β expression compared to those with only-WMHV infections, whereas there was no effect of co-infection in females. Additionally, WMHV-only males and

all females had higher *Tgf*-β expression when on lower-quality standard diets compared to those on higher-quality diets. Furthermore, there was no significant association between infection group and *Tgf*-β expression at day 7 (x^2_1=0.10, p=0.76; Table 5.3g) or day 35 (x^2_1=0.04, p=0.85; Table 5.3g). However, both diet (x^2_1=10.11, q=0.012) and sex (x^2_1=3.91, q=0.049) remained in the model at day 7 (Table 5.3i), with animals on standard quality diets having higher expression than those on high quality diets.

Additionally, IL-6 expression was significantly associated with infection group at day 7 (x^2_1=4.49, q=0.040), and there was a significant diet*sex interaction (x^2_1=5.78, q=0.032) (Table 5.3J, Figure 5.4d). Animals with co-infections had higher IL-6 expression compared to WMHV-only infected animals, and females on lower quality standard diets had higher expression than females on high quality diets. Conversely, there was no association between IL-6 expression and infection group at day 14 (x^2_1=1.68, p=0.19; Table 5.3K) or day 35 (x^2_1=0.001, p=0.97; Table 5.3L).

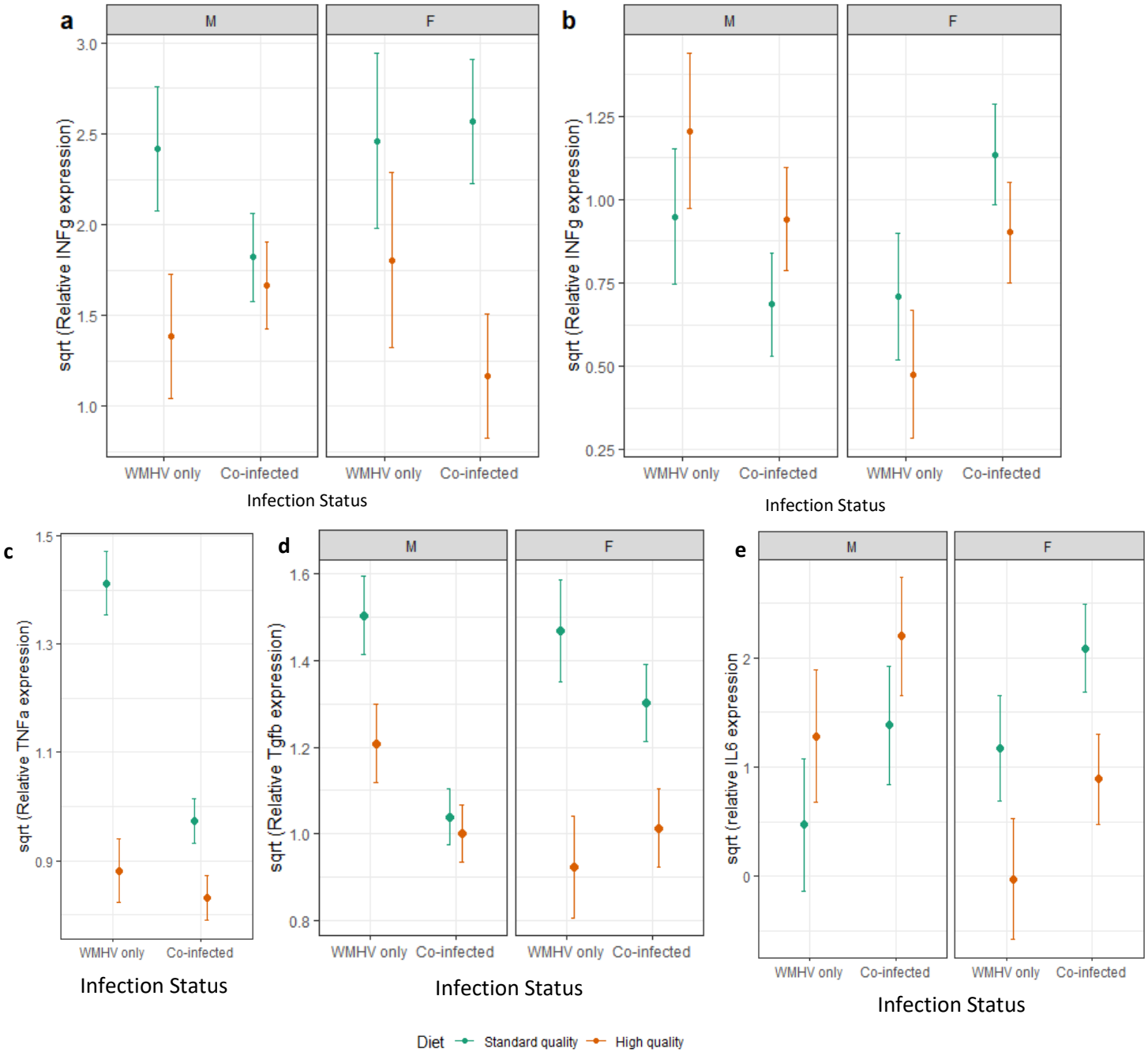

Figure 5.4. Association between **a)** INF-γ gene expression, sex, diet and infection group at day 7, **b)** INF-γ gene expression, sex, diet and infection group at day 14 , **c)** TNF-α gene expression, diet and infection group at day 14 **d)** TGF-β expression diet and infection group at day 14, e) IL-6 gene expression, diet, sex and infection group at day 7 in the spleen of laboratory bred wood mice (n=20). There is a very mixed response to co-infection, however the standard lower-quality diets increase Th1 expression compared to higher-quality diets, sometimes giving co-infected animals a boost of Th1 expression.

Table 5.3. Factors affecting Th1 cytokine expression levels in the spleens of laboratory bred wood mice. Models were simplified by backwards stepwise elimination. For variables not in the minimal model, statistics are those at the point that factor left the model. Q values indicate FDR-adjusted P-values (see Methods).

		A. *Inf*-γ day 7			**B.** *Inf*-γ day 14			**C.** *Inf*-γ day 35		
variable	df	X^2	p	q	X^2	p	q	X^2	p	q
infection group *Diet*Sex	1	0.0438	0.8342	-	4.0765	0.0435 ¥	0.0485	2.0297	0.1543	-
Diet*Sex	1	2.7882	0.0950	-	-	-	-	3.8722	0.0491 ¥	0.0491
infection group *Diet	1	0.0918	0.7619	-	-	-	-	0.1205	0.7285	-
infection group *Sex	1	0.0672	0.7955	-	-	-	-	6.1227	0.0134 ¥	0.0317
Diet	1	1.7346	0.1878	-	-	-	-	-	-	-
Sex	1	0.2444	0.6210	-	-	-	-	-	-	-
infection group	1	1.8028	0.1794	-	-	-	-	-	-	-
		D. *Tnf*-α day 7			**E.** *Tnf*-α day 14			**F.** *Tnf*-α day 35		
Variable	df	X^2	p	q	X^2	p	q	X^2	p	q
infection group *Diet*Sex	1	0.5713	0.4497	-	0.0050	0.9437	-	0.4654	0.4951	-
Diet*Sex	1	0.0149	0.9028	-	0.5102	0.4750	-	0.0416	0.8383	-
infection group *Diet	1	2.5067	0.1134	-	13.038	0.0003 ¥	0.0044	0.0002	0.9886	-
infection group *Sex	1	0.6843	0.4081	-	0.8563	0.3548	-	0.6786	0.4101	-
Diet	1	5.1124	0.0238 ¥	0.0333	-	-	-	0.2956	0.5866	-
Sex	1	0.2175	0.6410	-	0.0311	0.0817	-	1.6524	0.1986	-
infection group	1	0.0944	0.7587	-	-	-	-	0.0324	0.8571	-
		G. *Tgf*-β day 7			**H.** *Tgf*-β day 14			**I** *Tgf*-β day 35		
Variable	df	X^2	p		X^2	p		X^2	p	
infection group *Diet*Sex	1	1.4227	0.2330	-	3.5007	0.0613	-	0.7660	0.3815	-
Diet*Sex	1	3.7074	0.0542	-	4.9275	0.0264 ¥	0.0333	0.3598	0.5486	-
infection group *Diet	1	0.1255	0.7232	-	5.2058	0.0225 ¥	0.0333	0.1100	0.7402	-
infection group *Sex	1	0.5703	0.4502	-	6.0152	0.0142 ¥	0.0317	0.2827	0.5949	-
Diet	1	10.110	0.0015 ¥	0.0116	-	-	-	1.1385	0.2860	-
Sex	1	3.9050	0.0481 ¥	0.0491	-	-	-	0.0924	0.7611	-
infection group	1	0.0950	0.7580	-	-	-	-	0.0372	0.8470	-
		J. IL-6 day 7			**K.** IL-6 day 14			**L.** IL-6 day 35		
Variable	df	X^2	p	q	X^2	p	q	X^2	p	q
infection group *Diet*Sex	1	0.0131	0.9090	-	0.3934	0.5305	-	0.5988	0.4390	-
Diet*Sex	1	5.7842	0.0162 ¥	0.0319	0.0747	0.7846	-	2.6240	0.1053	-
infection group *Diet	1	1.4358	0.2308	-	0.7837	0.3760	-	0.9863	0.3207	-
infection group *Sex	1	0.3227	0.5699	-	0.3736	0.5411	-	3.8058	0.0511	-
Diet	1	-	-	-	1.7107	0.1909	-	0.7558	0.3847	-
Sex	1	-	-	-	0.8908	0.3453	-	0.0213	0.8841	-
infection group	1	4.4899	0.0341 ¥	0.0396	1.6809	0.1948	-	0.0012	0.9724	-
¥ Variables in the minimal model (P < 0.05)										

Type 2/immunoregulatory cytokines in the spleen

There was no significant association between infection group and IL-10 at any time point (Day 7: x^2_1=0.52, p=0.47, Table 5.4a; Day 14: x^2_1=0.29, p=0.59, Table 5.4b; Day 35: x^2_1=0.24, p=0.62, Table 5.4c). However, there was a significant diet*sex interaction at day 7 (x^2_1=7.68, q=0.023; Table 5.4a), showing that females on standard diets had higher IL-10 expression than females on high quality diets, and diet had no effect on IL-10 expression in males.

In contrast to IL10, IL-5 had a significant infection group *diet*sex interaction at day 7 (x^2_1=3.89, q=0.049; Table 5.4d, Figure 5.5a), day 14 (x^2_1=9.23, q=0.012; Table 5.4e, Figure 5.5b) and day 35 (x^2_1=6.29, q=0.031; Table 5.4f, Figure 5.5c). At day 7 co-infected females on standard diets had higher IL-5 expression than WMHV-only infected females on the same diet, whereas there was no effect of infection group on females on standard quality diets and mice on high quality diets. At day 14 co-infected females on standard diets and co-infected males on high quality diets had higher IL-5 expression compared to mice with WMHV-only infections, and there was no effect of co-infection on females on high quality diets and males on standard diets. At day 35 co-infected females on standard diets had higher IL-5 compared to WMHV-only females on the same diet, in contrast co-infected males on standard quality diets had higher IL5 expression compared to those WMHV-only infections. Additionally, WMHV-only males at day 7 and 14 and co-infected females at day 7, 14 and 35 on lower-quality standard diets had increased IL-5 expression compared to those on higher quality diets.

Table 5.4. Factors affecting Th2/immunoregulatory cytokine expression levels in the spleens of laboratory bred wood mice. Models were simplified by backwards stepwise elimination. For variables not in the minimal model, statistics are those at the point that factor left the model. Q values indicate FDR-adjusted P-values (see Methods).

		A. IL-10 day 7			**B.** IL-10 day 14			**C.** IL-10 day 35		
variable	df	X^2	p	q	X^2	p	q	X^2	p	q
infection group *Diet*Sex	1	1.4409	0.2300	-	0.8508	0.3563	-	0.3864	0.5342	-
Diet*Sex	1	7.6757	0.0056 ¥	0.0232	3.2426	0.0718	-	3.1997	0.0737	-
infection group *Diet	1	2.6658	0.1025	-	0.2673	0.6052	-	0.4457	0.5044	-
infection group *Sex	1	0.3642	0.5462	-	0.8259	0.3635	-	1.1496	0.2836	-
Diet	1	-	-	-	1.4368	0.2307	-	0.0008	0.9772	-
Sex	1	-	-	-	1.3212	0.2504	-	0.1512	0.6974	-
infection group	1	0.5167	0.4722	-	0.2886	0.5912	-	0.2429	0.6221	-
		D. IL-5 day 7			**E.** IL-5 day 14			**F.** IL-5 day 35		
Variable	df	X^2	p		X^2	p		X^2	p	
infection group *Diet*Sex	1	3.8867	0.0487 ¥	0.0491	9.2333	0.0024 ¥	0.0139	6.2892	0.0122 ¥	0.0317
Diet*Sex	1	-	-	-	-	-	-	-	-	-
infection group *Diet	1	-	-	-	-	-	-	-	-	-
infection group *Sex	1	-	-	-	-	-	-	-	-	-
Diet	1	-	-	-	-	-	-	-	-	-
Sex	1	-	-	-	-	-	-	-	-	-
infection group	1	-	-	-	-	-	-	-	-	-

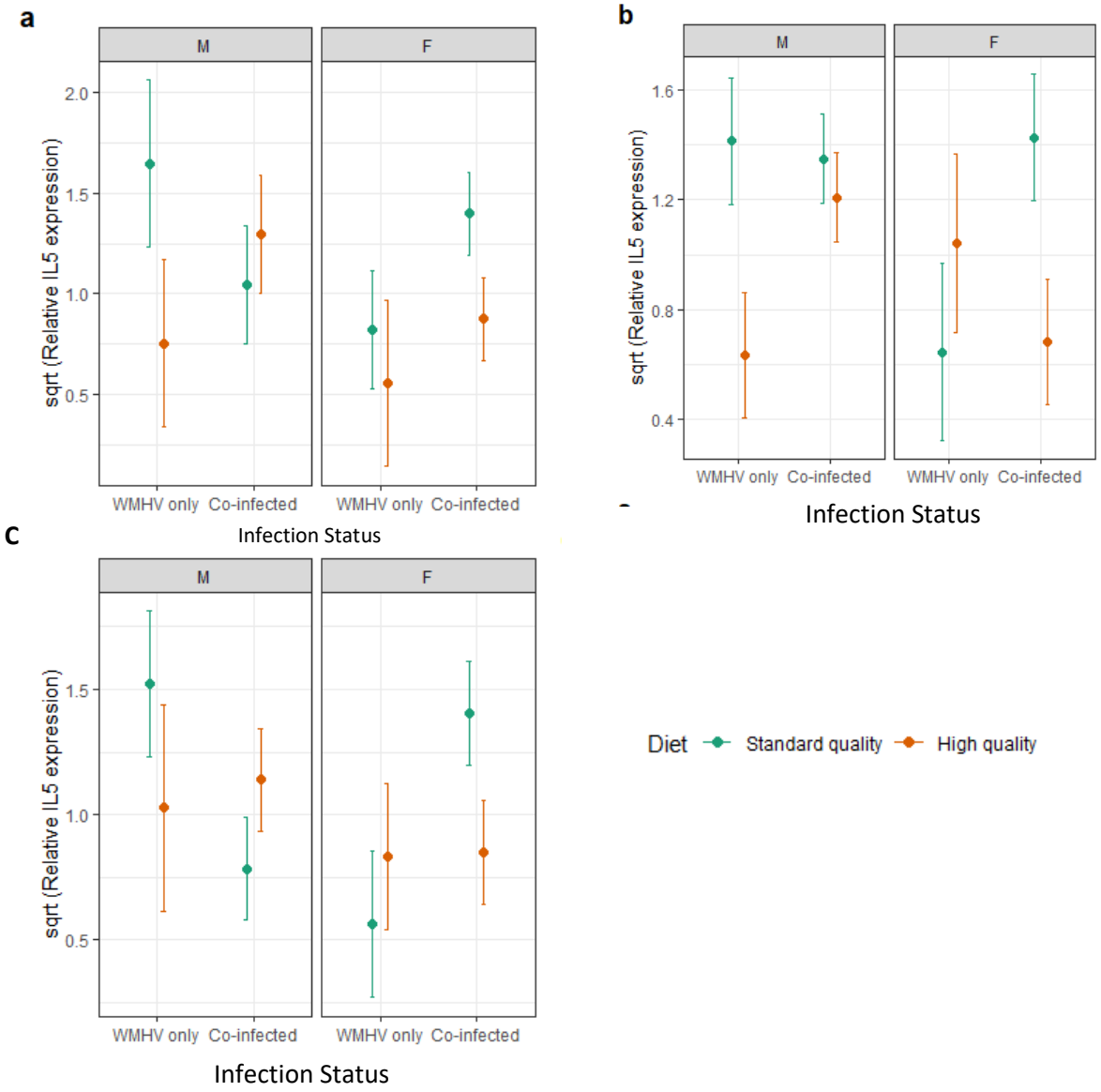

Figure 5.5. Association between **a)** IL-5 expression, diet and infection group at day 7, **b)** IL-5 expression, diet and infection group at day 14, **c)** IL-5 expression, diet and infection group at day 35; (n=20). Overall a mixed response to co-infection, although there seems to be more of a trend showing an increase in Th2 expression in co-infected mice compared to WMHV-only infected mice mainly in mice on lower-quality standard diets.

5.5.4 *Does H. bakeri co-infection downregulate the expression of cytokines aimed at WMHV infection in the lungs?*

Type 1 cytokines in the lung

There was no association between infection group and *Inf*-γ at day 7 (x^2_1=1.53, p=0.22; Table 5.5a) or day 35 (x^2_1=0.002, p=0.96; Table 5.5c). However, at day 14 an infection group *diet interaction remained in the model (x^2_1=5.44, q=0.032; Table 5.5b, Figure 5.6a). Co-infected mice on high quality diets had reduced *Inf* -γ expression than those with only

WMHV infection on the same diet. There was no effect of co-infection on *Inf* -γ expression in mice on standard quality diets.

For *Tnf*-α, there was no association between infection group and *Tnf* -α expression at day 7 (x^2_1=0.28, p=0.60; Table 5.5d), day 14 (x^2_1=0.35, p=0.55; Table 5.5e) or day 35 (x^2_1=0.001, p=0.38; Table 5.5f). However, there was a significant main effect of diet at day 7 (x^2_1=10.00, q=0.012; Table 5.5d), day 14 (x^2_1=5.46, q=0.032; Table 5.5e) and day 35 (x^2_1=4.78, q=0.035; Table 5f), with animals on standard quality diets having higher *Tnf* -α compared to those on high quality diets across all time points.

There was no significant association between infection group and *Tgf*- β at day 7 (x^2_1=0.0001, p=0.98; Table 5.5g), day 14 (x^2_1=1.42, p=0.23; Table 5.5h), or day 35 (x^2_1=0.04, p=0.83; Table 5i). However, there was a significant effect of sex at day 7 (x^2_1=5.56, q=0.032; Table 5.5g) and diet at day 35 (x^2_1=5.09, q=0.033; Table 5.5i), with higher *Tgf* -β expression in those on standard quality diets compared to high quality diets.

For IL-6, there was a significant infection group *diet*sex interaction at day 7 (x^2_1=30.43, q=0.003; Table 5.5J, Figure 5.6b), day 14 (x^2_1=6.41, q=0.032; Table 5.5K, Figure 5.6c) and day 35 (x^2_1=5.43, q=0.032; Table 5.5L, Figure 5.6d). At day 7, co-infected females on standard quality diets and males on high quality diets had reduced IL-6 expression, whereas co-infected males on standard quality diets had increased IL-6, and co-infection had no effect on females on high quality diets. At day 14, IL-6 expression was increased in co-infected standard quality females but decreased in co-infected males on high quality diets, and there was no effect of co-infection for males on standard quality diets or for females on high quality diets. Lastly, at day 35, co-infection was only associated with IL-6 expression in those on standard quality diets, with co-infected males had lower IL-6 expression while co-infected females had higher IL-6 expression.

Table 5.5. Factors affecting Th1 cytokine expression levels in the lungs of laboratory bred wood mice. Models were simplified by backwards stepwise elimination. For variables not in the minimal model, statistics are those at the point that factor left the model. Q values indicate FDR-adjusted P-values (see Methods).

		A. *Inf*-γ day 7			**B.** *Inf*-γ day 14			**C.** *Inf*-γ day 35		
variable	df	X^2	p	q	X^2	p	q	X^2	p	q
infection group *Diet*Sex	1	1.5275	0.2165	-	0.0135	0.9074	-	0.0020	0.9642	-
Diet*Sex	1	0.1527	0.6959	-	1.8778	0.1706	-	2.7539	0.0970	-
infection group *Diet	1	1.0732	0.3002	-	5.4366	0.0197 ¥	0.0319	0.0041	0.9488	-
infection group *Sex	1	0.6264	0.4287	-	0.7571	0.3843	-	0.0081	0.9285	-
Diet	1	2.8805	0.0897	-	-	-	-	0.5049	0.4774	-
Sex	1	1.7688	0.0850	-	0.2070	0.6491	-	1.2428	0.2649	-
infection group	1	0.1586	0.6904	-	-	-	-	2.1469	0.1429	-
		D. *Tnf*-α day 7			**E.** *Tnf*-α day 14			**F.** *Tnf*-α day 35		
Variable	df	X^2	p	q	X^2	p	q	X^2	p	q
infection group *Diet*Sex	1	0.1574	0.6915	-	0.3848	0.5350	-	2.2972	0.1296	-
Diet*Sex	1	2.1897	0.1389	-	0.0033	0.9539	-	3.0575	0.0803	-
infection group *Diet	1	0.0290	0.8648	-	3.5315	0.0602	-	3.4403	0.0636	-
infection group *Sex	1	2.3913	0.1220	-	0.2760	0.5994	-	0.0623	0.8029	-
Diet	1	9.9998	0.0016 ¥	0.0116	5.4618	0.0194 ¥	0.0319	4.7834	0.0287 ¥	0.0347
Sex	1	2.3028	0.1291	-	0.8259	0.3635	-	0.6453	0.4218	-
infection group	1	0.2816	0.5956	-	0.3527	0.5526	-	0.0008	0.9768	-
		G. *Tgf*-β day 7			**H.** *Tgf*-β day 14			**I.** *Tgf*-β day 35		
Variable	df	X^2	p	q	X^2	p	q	X^2	p	q
infection group *Diet*Sex	1	1.4977	0.2210	-	2.1345	0.1440	-	0.8000	0.3711	-
Diet*Sex	1	0.2803	0.5965	-	0.7841	0.3759	-	0.9275	0.3355	-
infection group *Diet	1	0.8130	0.3672	-	2.1267	0.1448	-	1.2917	0.2557	-
infection group *Sex	1	0.1103	0.7398	-	0.0014	0.9702	-	1.5527	0.2127	-
Diet	1	3.2024	0.0735	-	1.3344	0.2480	-	5.0876	0.0241 ¥	0.0333
Sex	1	5.5574	0.0184 ¥	0.0319	1.1645	0.2805	-	0.2920	0.5889	-
infection group	1	0.0008	0.9771	-	1.4163	0.2340	-	0.0449	0.8322	-
		J. IL-6 day 7			**K.** IL-6 day 14			**L.** IL-6 day 35		
Variable	df	X^2	p	q	X^2	p	q	X^2	p	q
infection group	1	30.4290	<0.0001 ¥	0.0029	6.4144	0.0113 ¥	0.0317	5.4338	0.0198 ¥	0.0319
infection group *Diet*Sex										
Diet*Sex	1	-	-	-	-	-	-	-	-	-
infection group *Diet	1	-	-	-	-	-	-	-	-	-
infection group *Sex	1	-	-	-	-	-	-	-	-	-
Diet	1	-	-	-	-	-	-	-	-	-
Sex	1	-	-	-	-	-	-	-	-	-
infection group	1	-	-	-	-	-	-	-	-	-

¥ Variables in the minimal model ($P < 0.05$)

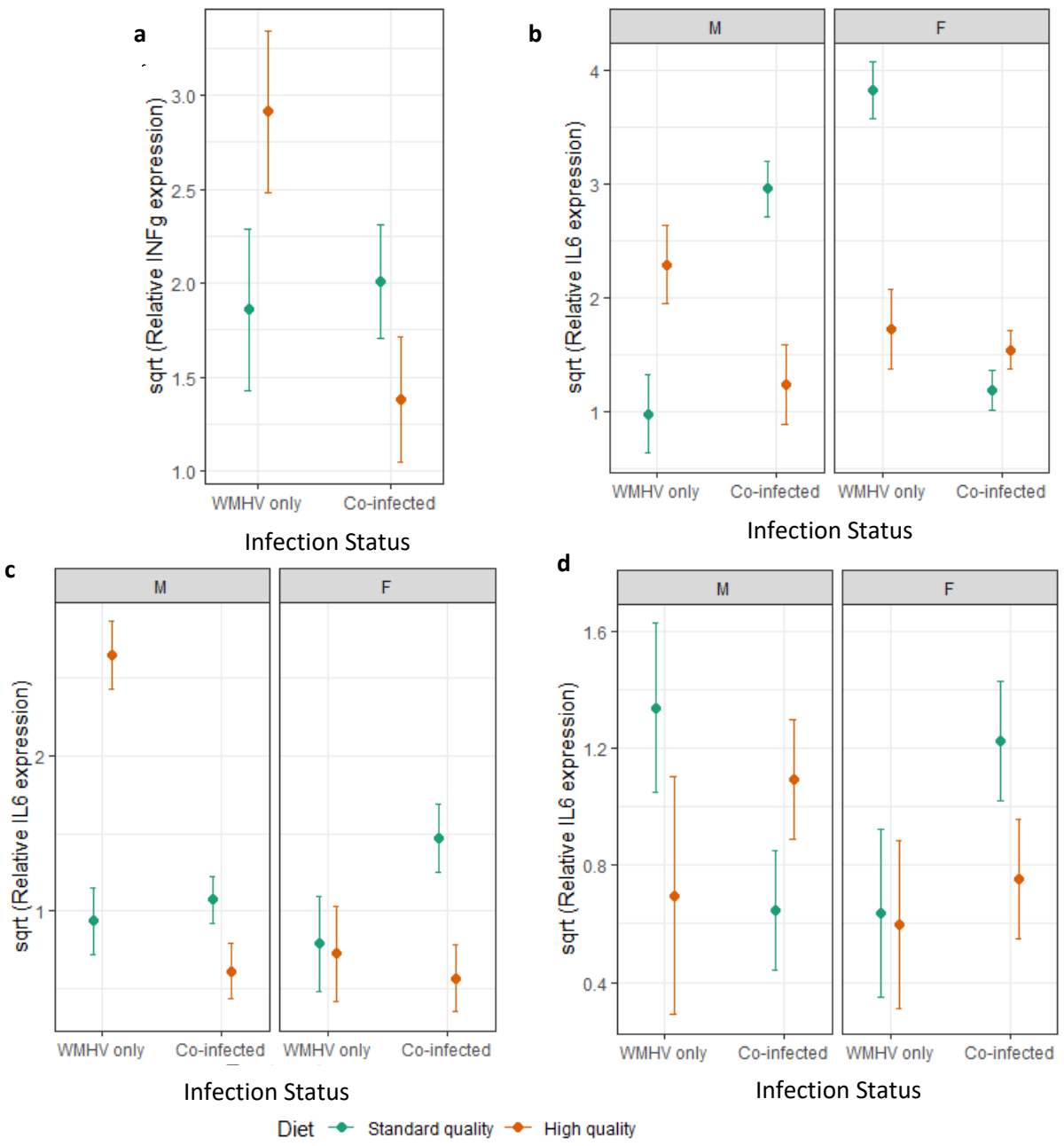

Figure 5.6. a) infection group , diet and *Inf*-γ expression at day 14 **b)** infection group , diet, sex and IL-6 expression at day 7, **c)** infection group , diet, sex and IL-6 expression at day 14, **d)** infection group , diet, sex and IL-6 expression at day 35 in the lungs of laboratory bred wood mice (n=20).

Type 2/immunoregulatory cytokines in lung

At day 7, IL-10 had a significant diet*sex*infection group interaction (x^2_1=6.05, q=0.032; Table 5.6a, Figure 5.7a). Co-infected males on standard quality diets had increased IL-10 expression compared to WMHV-only infected mice, however this could be overcome by a higher quality diet with co-infected males on high quality diets having lower IL-10 expression than those on standard quality diets. Additionally, co-infection had no effect on expression in all females or males on high quality diets. At day 14, there was a significant infection group *diet interaction (x^2_1=6.26, q=0.023; Table 5.6b, Figure 5.7b), showing that co-infected animals on high quality diets had lower IL-10 expression compared to WMHV-only infected mice on the same diet. Additionally, among those with co-infections higher quality diets reduced IL-10 expression compared to those on lower quality standard diets, and there was no effect of co-infection in mice on standard quality diets. Additionally, there was no association between IL-10 and infection group at day 35 (x^2_1=1.57, p=0.21; Table 5.7c).

For IL-5 infection group was not significantly associated with expression at day 7 (x^2_1=1.08, p=0.30; Table 5.6g) or day 35 (x^2_1=1.52, p=0.22; Table 5.6i) with only diet remaining in the model at day 7 (x^2_1=5.06, 0.033; Table 5.6g). At day 7 animals on standard quality diets had higher IL-5 expression compared to those on high quality diets. Conversely, there was a significant infection group *diet*Sex interaction at day 14 (x^2_1=7.86, 0.023; Table 5.6h, Figure 5.7c). Co-infected females on standard quality diets and co-infected males on high quality diets had higher IL-5 expression compared to those with WMHV-only infections, and that there was no effect of co-infection on males on standard quality diets and females on high quality diets.

Table 5.6. Factors affecting type 2/immunoregulatory cytokine expression levels in the lungs of laboratory bred wood mice. Models were simplified by backwards stepwise elimination. For variables not in the minimal model, statistics are those at the point that factor left the model. Q values indicate FDR-adjusted P-values (see Methods).

		A. IL-10 day 7			**B.** IL-10 day 14			**C.** IL-10 day 35		
variable	df	X^2	p	q	X^2	p	q	X^2	p	q
infection group *Diet*Sex	1	6.0518	0.0139 ¥	0.0317	0.9567	0.3280	-	0.3948	0.5298	-
Diet*Sex	1	-	-	-	1.1922	0.2749	-	1.5173	0.2180	-
infection group *Diet	1	-	-	-	6.2602	0.0124 ¥	0.0317	1.0697	0.3010	-
infection group *Sex	1	-	-	-	2.0324	0.1540	-	0.9096	0.3402	-
Diet	1	-	-	-	-	-	-	3.0322	0.816	-
Sex	1	-	-	-	1.6225	0.2028	-	0.1646	0.6849	-
infection group	1	-	-	-	-	-	-	1.5724	0.2099	-
		D. IL-5 day 7			**E.** IL-5 day 14			**F.** IL-5 day 35		
Variable	X^2	p	X^2	q	X^2	p	q	X^2	p	q
infection group *Diet*Sex	1	1.2967	0.2548	-	7.8610	0.0051 ¥	0.0232	1.6491	0.1991	-
Diet*Sex	1	0.0158	0.9001	-	-	-	-	1.9284	0.1649	-
infection group *Diet	1	0.1037	0.7474	-	-	-	-	2.1227	0.1451	-
infection group *Sex	1	0.4459	0.5043	-	-	-	-	0.6103	0.4347	-
Diet	1	5.0606	0.0245 ¥	0.0333	-	-	-	3.5809	0.0585	-
Sex	1	0.7178	0.3969	-	-	-	-	0.2504	0.6168	-
infection group	1	1.0783	0.2991	-	-	-	-	1.5235	0.2171	-

¥ Variables in the minimal model ($P < 0.05$)

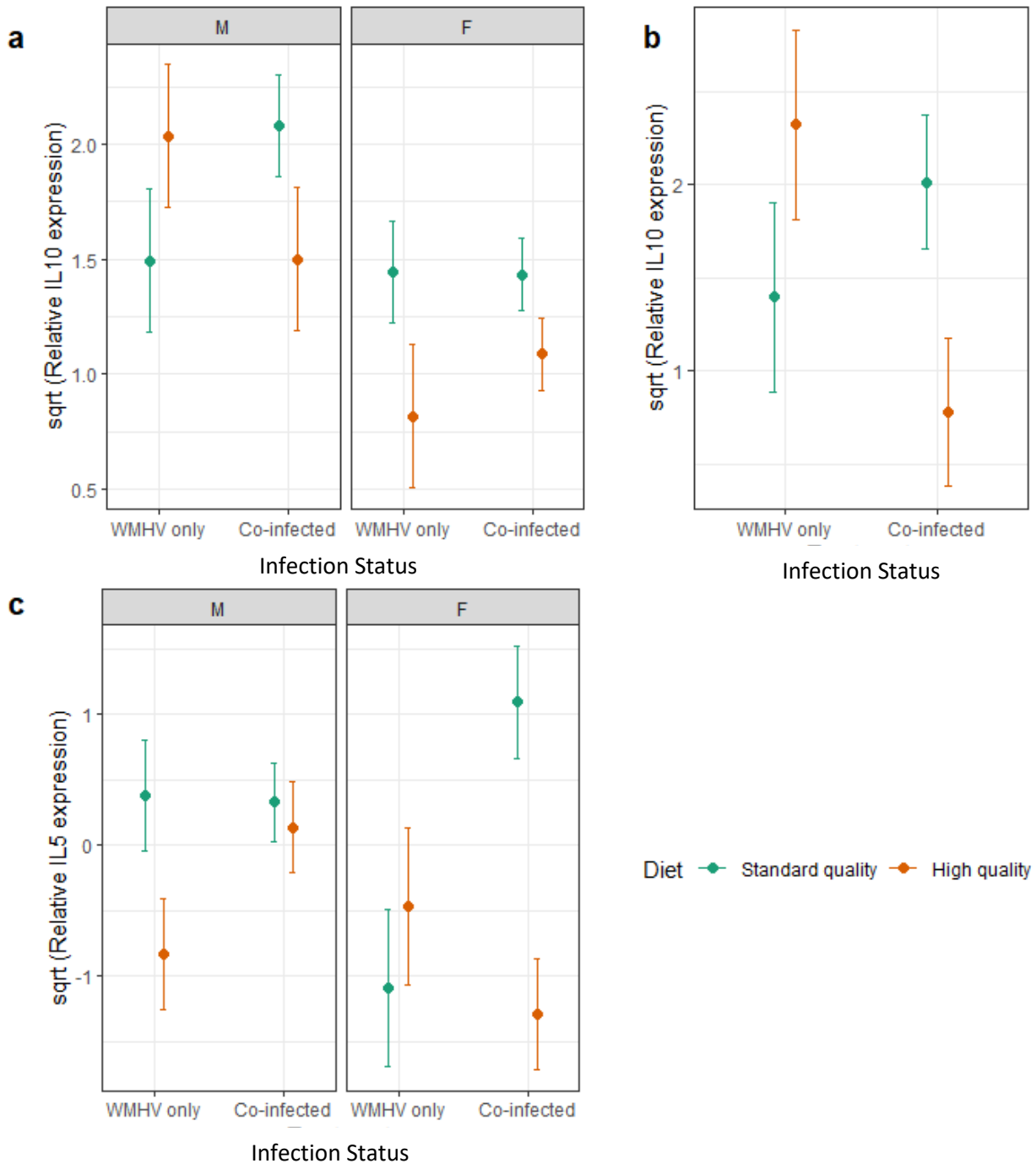

Figure 5.7. Association between **a)** treatment, sex, diet and IL-10 expression at day 7, **b)** treatment, diet and IL-10 expression at day 14, **c)** treatment, sex, diet and IL-5 expression at day 14 in the lungs of laboratory bred wood mice (n=20).

Overall, most of the effects of co-infection seen in mice on the lower quality standard diet were not observed in mice on the higher quality diets (figure 5.9). There is more of an effect of co-infection on cytokine expression in females on standard quality diets compared to females on high quality diets. However, in both males and females there is no clear increase/decrease in Th1 or Th2 expression in mice on either diet, even so there is more of a tendency for Th1 and Th2 expression to be decreased in males, while females on standard quality show more of an increase in expression compared to WMHV-only infected mice.

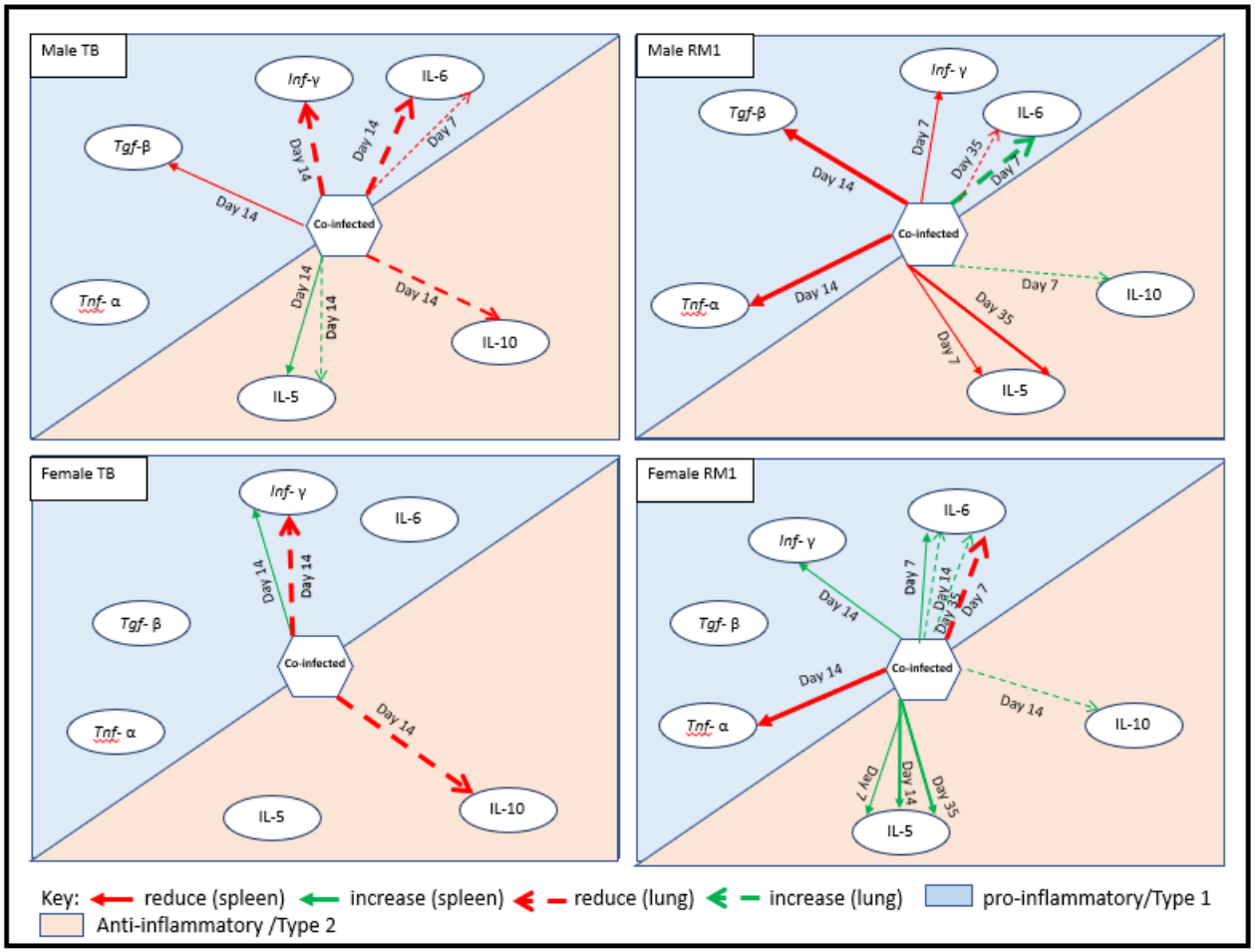

Figure 5.9. The effect of *H. bakeri* co-infection on cytokine gene expression in the spleens and lungs of laboratory bred wood mice compared to WMHV-only infected mice, for male and females on high quality or lower quality standard diets. Line thickness indicates effect size. Red arrows indicate that co-infection reduces expression compared to WMHV-only infected mice. Green arrows indicate that co-infection increases cytokine gene expression compared to WMHV-only infected mice. A light blue background shows pro-inflammatory/Type 1 cytokines while a light orange background shows anti-inflammatory/Type 2 cytokines.

Discussion

5.5.1. Effect of co-infection on the probability of having a detectable WMHV infection

I found that co-infection with *H. bakeri* had the same effect on the probability of having a detectable WMHV infection in both the lungs and the spleen. Only mice on lower quality standard diets were affected by *H. bakeri* co-infection, with co-infected mice less able to suppress WMHV infection below detectable levels at 7 and 35 dpi, compared to WMHV-only mice. These results suggest that *H. bakeri* co-infection reduces the host's ability to control WMHV infections, but that mice on high quality diets are able to over-come the suppressive effects of *H. bakeri* co-infection. This is not supprising since nutrition is essential for maintaining a correctly functoning immune system and since mice in the wild are unlikely to be reciving such a high quality diet, the lower quality standard diet is most likely to be indicatve of what we would expect to see in wild populations . Previous studies have found that co-infected mice are less able to control co-infecting viral infections. For example, mice infected with a recombinant vaccinia virus expressing HIV-1, took three weeks longer to clear the virus when co-infected with *Schistosoma mansoni,* compared to when infecting alone (Actor et al. 1993*).* Another study by Edwards et al. (2005) found that the liver of mice co-infected with *S. mansoni* and lymphocytic choriomeningitis virus (LCMV) was more susceptible to virus replication, compared to those only infected with LCMV. Additionally, previous studies have shown that nutrition can impact a host's ability to respond to infections (Cypher and Frost 1999, Coop and Kyriazakis 2001, Ezenwa 2004). For example, kit foxes from residential areas in California who therefore had access anthropogenic food sources, were found to be heavier and in better condition than animals in a reserve, and consequently had better immune status (Cypher and Frost 1999).

5.5.2. Effect of co-infection on WMHV lytic gene (ORF50) expression

To further determine the effects of co-infection on WMHV I analysed the expression of the latent-to-lytic switch gene, *ORF*50. I found that co-infection only affects *ORF*50 expression in the lungs and not in the spleen, which is of particuar importance since the lungs are the main site of lytic viral replication (Flaño et al. 2005, Hughes et al. 2010b). I found co-infected mice were more likely to still have detectable *ORF*50 expression in the lungs at 35 dpi on

both diets, whereas WMHV-only mice were able to clear lytic virus infection by 35 days post infection. This suggests that co-infected mice are less able to control viral replication, and so have lytic infections for longer than WMHV-only mice, potentially due to *H. bakeri* infection inducing a Th2 response within the host causing the anti-viral Th1 arm of the immune system to be downregulated. Indeed, Reese et al. (2014) found that helminth infection, characterized by induction of the cytokine IL-4, caused on-going murine γ-herpesvirus infection to become reactivated from latency *in vivo*. IL-4 promoted viral replication by binding to and acting on a viral promoter to induce expression of the viral latent-to-lytic switch gene (*ORF*50), and blocked the antiviral effects of INF-γ (Reese et al. 2014). These results could have consequences for WMHV infection dynamics since WMHV is only transmittable to others in the lytic stage of infection, meaning those with co-infections could be able to trasnmit the virus for longer periods of time compared to those with WMHV-only infections.

In contrast to results from previous chapters (chapters 2 and 3), and indeed to considerable evidence that helminth co-infection reduces the ability of the host to deal with co-infecting viral infections (Reese et al. 2014, Dietze et al. 2016), I found that among mice with detectable *ORF*50 expression, co-infected mice had lower expression of *ORF*50 levels at 7 and 14 dpi compared to WMHV-only infected mice. Hence, although co-infected mice were more likely to have lytic infections, those active infections had lower levels of *ORF*50 gene expression. This is a supprising result, because if co-infection with *H. bakeri* makes the host more susceptible to WMHV, It would be expected that expression levels of *ORF*50 would be higher in co-infected mice. For instance, helminth-HIV co-infection was associated with higher plasma HIV viral loads comared to inviduals without helminth co-infections (Mulu et al. 2013). However, our results suggest the opposite, in that during the lytic infection stage in the lungs (7 and 14 dpi) helminth co-infection seems to have a protective effect by reducing *ORF*50 expression levels compared to WMHV-only mice. This relationship then switches by 35 dpi, whereby only mice with co-infections had any detectable *ORF*50 expression and those with WMHV-only infections were able to clear lytic viral infection. A somewhat similar result was found recetly by Lin et al. (2019), who found that mice previously infected with *H. polygyrus* (mice were infected and then drug-cured 2 weeks later) were more likely to survive after challenge with the bacteria, *Listeria monocytogenes,*

compared to those only infected with *L. monocytogenes.* Additonally they found that *H. polygyrus* co-infetced mice had reduced bacterial burdens compared to mice only *L. monocytogenes* infections. There have also been other studies which show helminth infection offering protection. For example, McFarlane et al. (2017) found that mice co-infected with *H. polygyrus* and respiratory syncytial virus (RSV) had reduced viral loads compared to mice with just RSV, and *H. polygyrus* infections coincided with the upregulated expression of type 1 INF in the gut and lungs. However, the mechanisms underlying these different effects of helminth-microparasite co-infections are poorly understood and could be specific for different infection models and different parasite combinations (Lin et al. 2019). Taken together these results suggest that *H. bakeri* co-infection could be having an affect on the host immune system, therefore allowing for the within-host interactions seen in our laboratoy bred wood mice.

5.5.3. *Course of WMHV infection*

As expected the course of WMHV infection was different between the lungs and the spleen. At 7 dpi in the in lungs there was a high probability of mice having a detectable infection (detectable *ORF*73 expression) and of these mice with detectable infections all had lytic infections (detectable *ORF*50 expression). Furthermore, by 14 dpi all mice had lytic infections and high expression levels, and by 35 dpi the probability of having a detectable infection declined as did expression levels of both genes. Whereas in the spleen, the probability of a detectable infection was low at 7 dpi as were expression levels of both genes. However, both detectable expression and expression levels peaked at day 14, corresponding with peak latent infection in the spleen, and by 35 dpi the probability of having a detectable infection had declined but remained higher compared to the lungs. This is similar to the patterns of infection previously found by Hughes et al. (2010b) for MHV-68 in wood mice and laboratory mice.

5.5.4. *Effect of co-infection on type 1 cytokine gene expression*

To determine the mechanism of interaction between WMHV and *H. bakerii*, I used RT-qPCR to quantify expression of several cytokine genes important for controlling both WMHV (type 1) and *H. bakeri* (type 2) infections (Figure 8) . Overall, the effect of co-infection on type 1

cytokine gene expression in the spleen was fairly mixed, mostly there was no effect of co-infection on type 1 expression, however co-infection could also increase (day 7 IL-6 and day 35 females INF-γ) or decrease (day 14 males *Inf*-γ) gene expression of type 1 cytokines in the spleen. However, the effect of diet was relatively clear, mice on low quality standard diets increased the expression of type 1 cytokines compared to high quality diets, and had increased type 1 expression in mice with co-infections (*Inf*-γ day 7 females, IL-6 day 7 females and *Tnf*-α) compared to co-infected mice on higher quality diets. In the lungs, the effect of co-infection and diet on type 1 cytokine expression is very mixed but tends to reduce (*Inf*-γ day 14 on high quality, IL-6 day 7 males on high quality and females on standard quality, IL-6 day 14 and 35 males standard quality) rather than increase (IL-6 day 14 males on standard quality, IL-6 day 14 and 35 females on standard quality) type 1 cytokine gene expression.

Additionally, at day 14 co-infection decreased the expression of the pro-inflammatory Th17 cytokine, *Tgf*-β in the spleen of male mice compared to WMHV-only infected males, and mice on lower quality standard diets tended to have higher expression of *Tgf*-β compared to mice on high quality diets, however this effect of diet was removed in co-infected males. Indeed, there was elevated type 1 responses in mice on lower quality diets compared to those on high quality diets, since *Tgf*-β is produced by Th17 cells and is important in inflammatory responses I would be expected that *Tgf*-β would also be increased.

As with the spleen, I found that lower quality standard quality diets can sometimes overcome the suppressive effects of co-infection by increasing Th1 expression in co-infected mice compared to co-infected mice on higher quality diets. Although many studies have found that the Th2 responses aimed at helminth infection can downregulate the Th1 response needed to control co-infecting microparasites (Actor et al. 1993, Spiegel et al. 2003, Babu et al. 2009, Osborne et al. 2014, Gazzinelli-Guimarães et al. 2017), others have shown that helminth co-infection has no effect on Th1 responses (Dietze et al. 2016, Ahmed et al. 2017). Specifically, Dietze et al. (2016) found that mice co-infected with Friend virus (FV) and the helminth *Litomosoides sigmodontis* had increased viral loads compared to mice with only FV infections, however Th1 cytokine responses were not affected by co-infection. Instead Increased viral loads were associated with reduced titres of neutralizing virus-specific antibodies (Dietze et al. 2016). These results, showing that helminth co-infection

can affect some virus specific immune responses while leaving Th1 cytokine responses unaffected, could explain why co-infected mice on standard quality diets are less able to suppress WMHV gene expression below detectable levels, and why all co-infected mice have lytic infections for longer than mice with WMHV-only infections, but there is hardly any suppression of Th1 cytokines. This suggests that these effects of co-infection could be mediated by the downregulation of other immune responses separate from Th1 cytokine expression.

5.5.5. *Effect of co-infection on type 2 cytokine gene expression*

Co-infection increased the expression type 2 cytokines (IL-5) in females on lower quality standard diets across all time points, while those on higher quality diets did not show any difference in expression between co-infected and WMHV-only infected mice. Males showed more of a mixed response, with co-infected males on high quality diets at 14 dpi showing increased expression and standard quality males at 35 dpi showing reduced expression. These results suggest that (particularly in females) higher quality diets can remove the effect of co-infection on IL-5 expression and can reduce expression in co-infected mice compared to co-infected mice on lower quality standard diets. In the lungs co-infection increases IL-5 at day 14 (high quality males and standard quality females) and mice on standard quality diets tend to have higher expression compared to mice on higher quality diets. These results suggest that lower quality diets are associated with higher levels of Th2 cytokines and are more likely to see co-infection increase Th2 cytokine gene expression. These results could partly explain why co-infection only reduces the ability of mice to suppress WMHV infection below detectable levels in mice on lower quality diets.

In the lungs I found that lower quality standard diets tended to increase IL-10 expression compared to those on high quality diets, giving co-infected mice a boost of IL-10 expression. The evidence indicates that co-infected mice have reduced expression of immunoregulatory cytokines compared to mice with WMHV-only infections, and that lower quality diets can increase expression of these cytokines. This effect of diet could be explained by the potential for low quality diets to increase pro-inflammatory responses (Ing et al. 2000).

Conclusion

Overall, I show that *H. bakeri* co-infection reduces the ability of the host to control WMHV in mice on lower quality diets. Interestingly, those on high quality diets showed no association between co-infection and ability to control WMHV infection suggesting that higher quality diets can overcome the negative effects of co-infection. Additionally, I found that being co-infected with *H. bakeri* was associated with mice having a longer period of lytic infection in the lungs, however during the early stages of WMHV infection, co-infection seems to enable to host to suppress lytic virus in the lungs to lower levels.

I expected that, since *H. bakeri* co-infection overall has a suppressive effect on the host's ability to control WMHV, I would see the downregulation of type 1 cytokine expression and upregulation of type 2 cytokine expression in co-infected mice. There was some evidence of co-infected mice having higher type 2 (IL-5) expression, however I found the type 1 response to co-infection was very mixed. This suggests that other mechanisms other than type 1 cytokine suppression could contribute to the reduced ability of the host to deal with WMHV.

6. Final discussion

6.1 Effect of co-infection on susceptibility to WMHV in a wild wood mouse population

One of the key aims of this thesis was to establish whether co-infection with *H. polygyrus* reduces the host's ability to deal with co-infecting WMHV, and if deworming treatments could change this outcome. Throughout this thesis, I found numerous occasions where *H. polygyrus* co-infection seems to hinder the host's ability to effectively deal with WMHV. In chapter 2, I found that among mice given prior deworming treatments, those with higher *H. polygyrus* FECs when treated were associated with an increased probability of having a WMHV infection 2-3 weeks later. It has previously been noted that FECs quickly return to pre-treatment levels in this system (Knowles et al. 2013, Clerc 2017). Hence, giving short-lived deworming treatments and having worm burdens bounce back as the effects of deworming wear off, has a detrimental effect on the host's ability to control WMHV. In fact, in this system deworming treatment (potentially due to its short-lived effects on *H. polygyrus* FECs) was not beneficial to the host in terms of reducing susceptibility to WMHV. In contrast, in chapter 3 there was evidence that mice given effective deworming treatments (~70% of treated mice were not infected with *H. polygyrus* when sacrificed and had a mean *H. polygyrus* FEC of 1 (compared to 19 for untreated mice)) were less likely to have a WMHV infection ~2 weeks after treatment (chapter 3). Furthermore, in chapter 2, the detrimental effect of helminth reinfection on the host's ability to control WMHV is exacerbated by higher *H. polygyrus* FECs, hinting that the suppressive effects of *H. polygyrus* are FEC dependent. Furthermore, I found that higher *H. polygyrus* burdens were associated with higher *ORF*73 gene expression, providing further evidence that effect of *H. polygyrus* can be burden dependent (chapter 3).

These findings that helminth co-infection can reduce the host's ability to respond to microparasitic infections has been demonstrated for many unnaturally clean SPF (Specific Pathogen Free) laboratory studies (Actor et al. 1993, Edwards et al. 2005, Su et al. 2014), but few have shown this in free living naturally infected animals. However, these few wild studies have found results similar to ours, in that helminth co-infection can increase

susceptibility to microparasites, and subsequent disease severity. For example, wild voles infected with *Heligmosomum mixtum* were more likely to become infected with Puumala Hantavirus infections (PUUV) (Guivier et al. 2014); wild boars co-infected with bovine tuberculosis (bTB) and a nematode species (*Metastrongylus* spp.) had an increased infection severity compared to those only infected with bTB (Risco et al. 2014), and African buffalo infected with helminths had an increased probability of becoming infected with tuberculosis (Ezenwa et al. 2010). To further explore the effects of helminth co-infection on WMHV, I also investigated whether co-infected mice are more likely to have a lytic (active, and transmissible) WMHV infection. It has been shown using *H. polygyrus* and MHV-68 in laboratory mice, which are not the natural host for these parasites, that *H. polygyrus* co-infection is able to reactivate MHV-68 from latency (Reese et al. 2014). In contrast to this I found in our wild wood mouse system that neither *H. polygyrus* co-infection nor ivermectin treatment was associated with having a lytic WMHV infection. These differing results could potentially show the importance of using natural host-parasite combinations, and of using 'dirty' mice with natural immune systems; laboratory mice raised in SPF conditions have been found to have immune systems more closely related to those of neonatal humans than adult humans (Beura et al. 2016a). Alternatively, it could be that our trapping timescales were not ideal to catch any effect of helminth co-infection on lytic virus gene expression, for example any effects could potentially only have been detectable before or after the final capture, or occur between successive trapping events. Furthermore, since I only analysed spleen samples from the natural population in chapter 3, and most lytic virus replication happens in the lungs, I would have missed any potential interactions that would only have been detectable in the lungs. Consequently, any further studies could use mice sacrificed at different time points post treatment and include analysis of lung samples, thus potentially capturing any interactions that happen in the lungs and spleen more or less than two weeks after treatment.

The results from chapters 2 and 3 could have implications for deworming programmes in locations where concurrent infection with multiple parasite species is common. With evidence mounting that helminth infections can have an impact on co-infecting microparasites, it has been questioned whether the severity of, and susceptibility to, infection with microparasites could be altered using anti-helminthic drugs (Nacher 2006).

Helminth infections can cause a considerable amount of disease burden in human populations in developing countries (Brooker 2010). Consequently, there have been calls for mass deworming to be introduced into control measures against major viral, bacterial or protozoal diseases (Molyneux et al. 2005, Hotez 2009). Our results suggest that deworming can reduce susceptibility to microparasitic infections, however any benefits could be quickly reversed if treatment is not long-lasting meaning worm burdens quickly return to pre-treatment levels. Additionally, our results highlight how treatment effects can differ even among the same host-parasite systems. In chapter 2, treatment had no beneficial effects on susceptibility to WMHV, however mice who had received treatment and had high *H. polygyrus* FECs were more susceptible to WMHV than their untreated counterparts. Conversely, in chapter 3 (where the study was carried out in a different population of mice from Falkirk, rather than Liverpool) I found that treatment was associated with reduced susceptibility to WMHV infection. This difference is likely to be due to study design, mice from Falkirk were treated with both ivermectin and Pytantel, whereas mice in Liverpool were treated less frequently and only with ivermectin. This is especially important as some studies have found that the anti-inflammatory effects of helminth infections can protect against immunopathology caused by microparasitic infections, and therefore be beneficial to the host (Bazzone et al. 2008).

6.2 *Effects of co-infection on the host immune response*

In terms of the mechanisms driving these co-infection interactions, helminths are widely known to have suppressive effects on the host immune system, causing immune-mediated within-host interactions during co-infection with microparasites such as viruses, bacteria or protozoa (Maizels et al. 2004, Elliott et al. 2007, Maizels et al. 2012a). These suppressive effects are most often attributed to the macroparasite fighting Th2 arm of the host immune system's ability to down regulate the microparasite fighting Th1 immune response. For example, Actor et al. (1993) infected mice that had previously been infected with *Schistosoma mansoni,* with a recombinant vaccinia virus expressing the HIV-1 protein gp120 and found that co-infected mice could take as much as 3 weeks longer to clear the virus. Furthermore, stimulated spleen cells from co-infected mice produced lower Th1 responses (INF- γ and IL-2) when challenged with gp120 than the vaccinia virus alone (Actor et al. 1993). Additionally, *H. polygyrus* co-infection initiated the reactivation of MHV-68 in

laboratory mice via induced Th2 responses (IL-4) and suppression of Th1 responses (INF-γ) (Reese et al. 2014). Hence, I hypothesised that the main drivers of *H. polygyrus*-WMHV interactions were immune mediated, via suppression of the Th1 response by the helminth induced Th2 response. I found that type 1 expression was lower in mice with current (TNF-α and INF-γ (albeit not statistically significant)) and previous (IL-6) *H. polygyrus* infections (chapter 4). However, type 2 expression was not higher in mice infected with *H. polygyrus* and TGF-β expression was higher in WMHV-only mice (chapter 4). While these results agree with the hypothesis that type 1 responses will be downregulated in co-infected mice, surprisingly I found that type 2 cytokine gene expression was not upregulated under helminth infection, compared to WMHV-only infected mice. This could be an artifact of sampling time scales, with our sampling missing the period when type 2 cytokines are upregulated. An alternative hypothesis could be that other immune mechanisms, or suppression directly caused by *H. polygyrus*, could be responsible for the downregulation of Th1 responses seen in co-infected mice. In fact, *H. polygyrus* is well known for excreting proteins which mimic the effects of TGF-β and can aid in the survival of *H. polygyrus* by downregulating the immune response (Johnston et al. 2017, Smyth et al. 2018).

6.3 *Effect of H. bakeri co-infection on WMHV in laboratory bred wood mice*

To better understand how co-infection effects WMHV and host Th1/Th2 responses I used an outbred colony of previously wild wood mice in controlled laboratory conditions (chapter 5). This allowed us to collect samples from specific time points post infection and eliminate other potential factors which could affect host immunity. Importantly, our 'wild-like' system has an advantage over inbred SPF-raised mice in other laboratory studies, since it is a wild-caught outbred colony and wood mice are the natural hosts of both WMHV and *H. polygyrus* meaning it is more likely to accurately capture the host-parasite interactions we would see in the wild. I was also able to give mice a standard quality and high quality diet to determine the effect of host nutritional status on co-infection effects. I found that mice on the lower quality diets were less able to control WMHV infection in the lungs and spleen than those on high quality diets, and co-infected mice on both diets were less able to suppress lytic virus in the lungs at day 35 post infection than single-infected mice. These results in the laboratory agree with my results from wood mice in the wild, in that co-infected mice are less able to control WMHV infections, and there is no effect of co-

infection on lytic virus in the spleen. Thus, this backs up the argument for analysing lung samples from wild wood mice to allow detection of any effects on lytic WMHV. It was also found that in the early stages of infection in the lungs (day 7 and 14 post infection), co-infected mice had lower quantities of *ORF*50 expression (the marker for lytic WMHV infection) suggesting that *H. bakeri* could be having a protective effect in the lungs during the early stages of infection. Helminths having protective effects (i.e. the host is better able to control microparasitic infection) has already been demonstrated in other studies (McFarlane et al. 2017, Lin et al. 2019). However, to fully understand the effects of co-infection on lytic WMHV it would be beneficial to perform plaque assays for lytic virus on lung tissues to determine accurate viral loads.

6.4 *Effect of H. bakeri co-infection on host immune repose in laboratory bred wood mice*

To further explore how helminth co-infection effects WMHV in our out-bred wood mouse colony, and how that compares to what we see in the wild, I analysed gene expression for several type 1, type 2 and immunoregulatory cytokines. I found that overall, there was no effect of co-infection on the expression of type 1 cytokines between co-infected and WMHV only infected mice. This is surprising since in the wild there was a decrease in type 1 expression in co-infected mice. Again, in contrast to what is seen in the wild this data suggests that the overall trend was for Th2 cytokine expression to be increased in co-infected mice.

6.5 *Effect of deworming on co-infecting WMHV*

I also found that co-infection effects were the same regardless of whether the mouse was given deworming treatments or not. One hypothesis for this could be that mice given deworming treatments had the same response to those not given treatments because in the lab environment mice are quickly able to clear *H. bakeri* infections, even after multiple doses. Indeed, in our system very few untreated mice had worms in the gut on dissection suggesting that they had cleared their infections without the need for deworming treatments. Alternatively, it could be that in this system any previous infection with *H. bakeri* has a lasting effect on the host immune system, regardless of whether the infection is cleared or not. These results differ somewhat from the results found in wild wood mice, where results suggested that effective treatment can reduce susceptibility to WMHV.

Explaining these differences requires an understanding of the differences between experiments in wild systems and those in laboratories. Wild animals will have a diverse history of previous infection whereas mice in the laboratory are immunologically naïve. Additionally, mice in the wild will experience many different stressors such as food shortage, adverse weather and predation which are not possible to replicate in the laboratory environment. Furthermore, mating behaviours in wild mice such as scent marking, male on male fighting and pregnancy could all affect susceptibility to microparasitic diseases. Indeed, I found in chapter 2 that heavier male mice were more at risk of WMHV infection, which agrees with results found by . All these results, taken together, show how variable the effects of deworming treatments can be even within the same host-parasite system. The benefits of deworming treatments for controlling microparasitic diseases are debated, mainly due to conflicting results on the outcomes of deworming co-infected individuals. For example, some studies show reduced HIV viremia (Mulu et al. 2013), and increased $CD4^+$ cell count (Blish et al. 2010) in anthelminthic treated individuals. Additionally, Wolday et al. (2002) studied the effect of anthelminthic treatment on HIV viral load in individuals co-infected with helminths and HIV in Ethiopia. They found that six weeks after treatment there was a significant change in HIV viral loads between treated and non-treated individuals- with treated individuals having lower viral loads. However, Modjarrad et al. (2005) found that there was no association between the treatment of helminth infections and reduced HIV viral load in adults in Zambia. Additionally, there is also evidence that interactions between helminths and microparasites can be negative (infection with one parasite infers resistance to another) (Pedersen and Fenton 2007). Hence, the outcome of treatment can be variable and any other potential factors such as nutritional status, behaviour (scent marking, male on male fighting etc) or adverse weather conditions should also be taken into account when assessing if deworming could be beneficial for controlling microparasitic diseases, particularly if evidence for or against comes from laboratory based studies.

6.6 *Diet effects on WMHV-H. bakeri co-infection dynamics*

The results from chapter 5 also suggest that high-quality diets can overcome many of the effects of co-infection. Previous studies have shown that nutrition can impact a host's ability to respond to infections (Cypher and Frost 1999, Coop and Kyriazakis 2001, Ezenwa 2004).

For example, kit foxes from residential areas in California who therefore had access to anthropogenic food sources, were found to be heavier and in better condition than animals in a reserve, and consequently had better immune status (Cypher and Frost 1999). In fact, our results show that increased nutrition could be better at overcoming the impact of helminth infection on the host's ability to respond to microparasitic co-infections than deworming. This could be particularly important when considering integrated control programmes for poor people in developing countries where helminth co-infections are common and can cause a considerable disease burden in human populations (Brooker 2010). The effects of nutritional status on the host immune response have been well studied. For example, energy deficits in laboratory mice have been shown to simultaneously suppress both Th1 and Th2 responses and their effector functions (Koski et al. 1999), and IL-4 was suppressed in the spleens of zinc deficient mice (Scott and Koski 2000). Whereas, protein malnutrition was associated with increased the survival of *H. polygyrus*, reduced levels the Th2 cytokine IL-4 and increased levels of the Th1 cytokine INF-g (Ing et al. 2000). There was a somewhat mixed response of Th2 cytokine gene expression to diet. Even so, there was a trend (especially in females) for mice on lower quality diets to have higher levels of IL-5 compared to mice on higher quality diets, and co-infection only increased IL-5 expression in mice on lower quality diets . Additionally, those on lower quality diets also had higher expression of the Th2/immunoregulatory cytokines (IL-10) and Type 1 cytokines (*Tgf*-β and, IL-6, *Inf*-γ) compared to mice on higher quality diets. These differences in immune response between the two diets could explain differing effects of co-infection between the diets. To further understand the effects of nutrition on co-infection interactions it would be beneficial to further study the effect of increased nutrition on co-infection dynamics in the wild, since there was some evidence that deworming can sometimes reduce susceptibility to microparasitic infections in wild wood mouse populations.

6.7 *Final conclusion*

Overall, *H. polygyrus* co-infection was found to hinder the host ability to deal with WMHV infections in the wild and in the laboratory. In the wild co-infection increased susceptibility to WMHV infection, higher *H. polygyrus* burdens were associated with increased *ORF*73 gene expression. Likewise, in the laboratory co-infection increased susceptibility to infection and was associated with higher expression of *ORF*73 (a proxy for viral load), and co-infected

mice were more likely to have longer lytic WMHV infections than singly infected mice. Despite this evidence that *H. polygyrus* infection can have a detrimental effect on the host's ability to deal with co-infecting WMHV, the effects of deworming treatments were variable. In the wild effective deworming treatment was associated with reduced susceptibility to WMHV infection, but short-lived deworming treatments (resulting in subsequent *H. polygyrus* re-infection) were associated with an increase in susceptibility to WMHV infection. Additionally, in the lab there was no effect of deworming treatments on susceptibility to infection or duration of lytic WMHV infections. These differing effects of deworming treatments between the wild and laboratory are most likely because animals in the lab can clear *H. bakeri* infections without treatment, thus most untreated mice had no *H. bakeri* infection upon sacrifice. Additionally, once infections were cleared there were no opportunities for reinfection as would be the case for wild wood mice. The effect of co-infection on Th1 and Th2 cytokine responses was somewhat mixed. In the wild co-infection was associated with reduced Th1 response but no effect on Th2 cytokine expression. Conversely, there was no association between co-infection and reduced Th1 responses in the lab, but there was a trend towards increased Th2/immunoregulatory responses in co-infected mice compared to WMHV-only infected mice. However, this study only measured the gene expression of cytokines and there will be varying levels of correlation between RNA and protein expression for each of the cytokine we measured. To get a better insight into how co-infection affects the immune response further work would need to be done on levels of Th1 and Th2 immune cells.

Taken together, these results show that co-infection with *H. polygyrus* has the potential to reduce the host ability to deal with co-infecting WMHV, and that effective deworming treatments have the potential to reverse this effect. Furthermore, these results show how complex within-host co-infection interactions can be and why it is particularly important to study these interactions in the natural host, since results can vary between the lab and the field even with the same, natural, host-parasite combinations. They also highlight that whilst the host immune response can play an important role in determining the outcomes of co-infections, other factors such as host nutritional status can also plays a critical role, which could potentially have implications if mass deworming was used to control major microparasitic infections. I also provided additional support to moving away from artificially

clean SPF laboratory studies, and instead using wild or 'dirty' lab animals, whose immune systems are challenged by co-infections and/or poor nutrition, and who are the natural hosts for the parasites. Thus, providing a more natural model of infection, with the hope that these results will more accurately capture what is seen in wild animal and human populations, thus enabling more successful treatment programmes.

7. Supplementary material

Table 7.1. Factors affecting WMHV gene expression levels in the spleen of laboratory bred wood mice infected with WMHV. Models were simplified by backwards stepwise elimination. For variables not in the minimal model, statistics are those at the point they left the model. The variable 'time' refers to the number of days since WMHV infection. infection group includes chronic *H. bakeri*, prior *H. bakeri* and WMHV-only

		A. *ORF*73 p/a (AIC: 60.76)		**B.** *ORF*73 quantity (AIC:206.37)	
Variable	df	X^2	p	X^2	p
Time*Diet* infection group	1	0.0000	1.0000	6.6648	0.0834
Time* infection group	2	4.7583	0.3130	0.3102	0.5408
Time*Diet	2	6.7004	0.0351 ¥	0.4851	0.7846
Time*Sex	2	3.0825	0.2141	0.9748	0.6142
Diet* infection group	1	6.2325	0.0443 ¥	0.6230	0.7324
Diet	1	-	-	0.6510	0.4197
Sex	1	5.8280	0.0158 ¥	0.1463	0.7021
infection group	1	-	-	0.8160	0.6649
Time	2	-	-	32.933	<0.0001 ¥
		C. *ORF*50 p/a (AIC: 71.62)		**D.** *ORF*50 quantity (AIC: 174.83)	
Variable	df	X^2	p	X^2	p
Time*Diet* infection group infection group	1	0.0000	1.0000	1.9701	0.3734
Time* infection group	2	1.3861	0.8466	1.5428	0.9724
Time*Diet	2	1.4017	0.4962	1.0391	0.5948
Time*Sex	2	0.8320	0.6597	1.1142	0.5729
Diet* infection group	1	3.9330	0.1400	0.2341	0.8896
Diet	1	1.4260	0.2325	0.0110	0.3146
Sex	1	2.5730	0.1087	4.0290	0.0447 ¥
infection group	1	1.4680	0.4800	1.6360	0.4413
Time	2	29.212	<0.0001 ¥	31.907	<0.0001 ¥
¥ Variables in the minimal model (P < 0.05)					

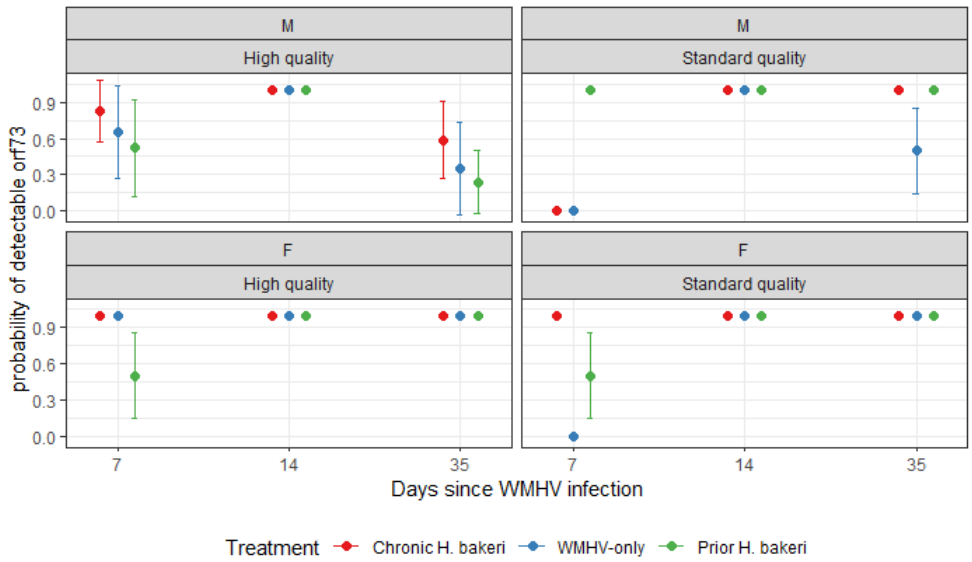

Figure 6.1. probability WMHV infection (detectable *ORF*73 expression) diet, sex and infection group , and days since WMHV infection in the spleens of laboratory bread wood mice.

Table 7.2. Factors affecting WMHV gene expression levels in the lungs of laboratory bred wood mice infected with WMHV. Models were simplified by backwards stepwise elimination. For variables not in the minimal model, statistics are those at the point they left the model. The variable 'time' refers to the number of days since WMHV infection. infection group includes chronic *H. bakeri*, prior *H. bakeri* and WMHV-only

		A. *ORF*73 p/a (AIC: 50.32)		**B.** *ORF*73 quantity (AIC:159.14)	
Variable	df	X^2	p	X^2	p
Time*Diet* infection group	1	0.0000	1.0000	6.4377	0.1688
Time* infection group	2	6.0309	0.0490 ¥	7.2185	0.1248
Time*Diet	2	4.4987	0.1055	3.5257	0.1716
Time*Sex	2	0.0000	1.0000	2.5528	0.2790
Diet* infection group	1	4.8917	0.2986	2.1581	0.3399
Diet	1	-	-	0.0983	0.7539
Sex	1	20.555	<0.0001 ¥	2.0923	0.1480
infection group	1	-	-	1.5303	0.4653
Time	2	23.5974	<0.0001 ¥	12.044	0.0024 ¥
		C. *ORF*50 p/a (AIC: 57.99)		**D.** *ORF*50 quantity (AIC: 117.06)	
Variable	df	X^2	p	X^2	p
Time*Diet* infection group	1	0.0000	1.0000	9.4829	0.0235 ¥
Time* infection group	2	0.0000	1.0000	-	-
Time*Diet	2	0.0000	1.0000	-	-
Time*Sex	2	0.0000	1.0000	6.0410	0.0488 ¥
Diet* infection group	1	1.5140	0.4692	-	-
Diet	1	0.0000	1.0000	-	-
Sex	1	4.5570	0.0328 ¥	-	-
infection group	1	6.3890	0.0410	-	-
Time	2	51.663	<0.0001 ¥	-	-
¥ Variables in the minimal model (P < 0.05)					

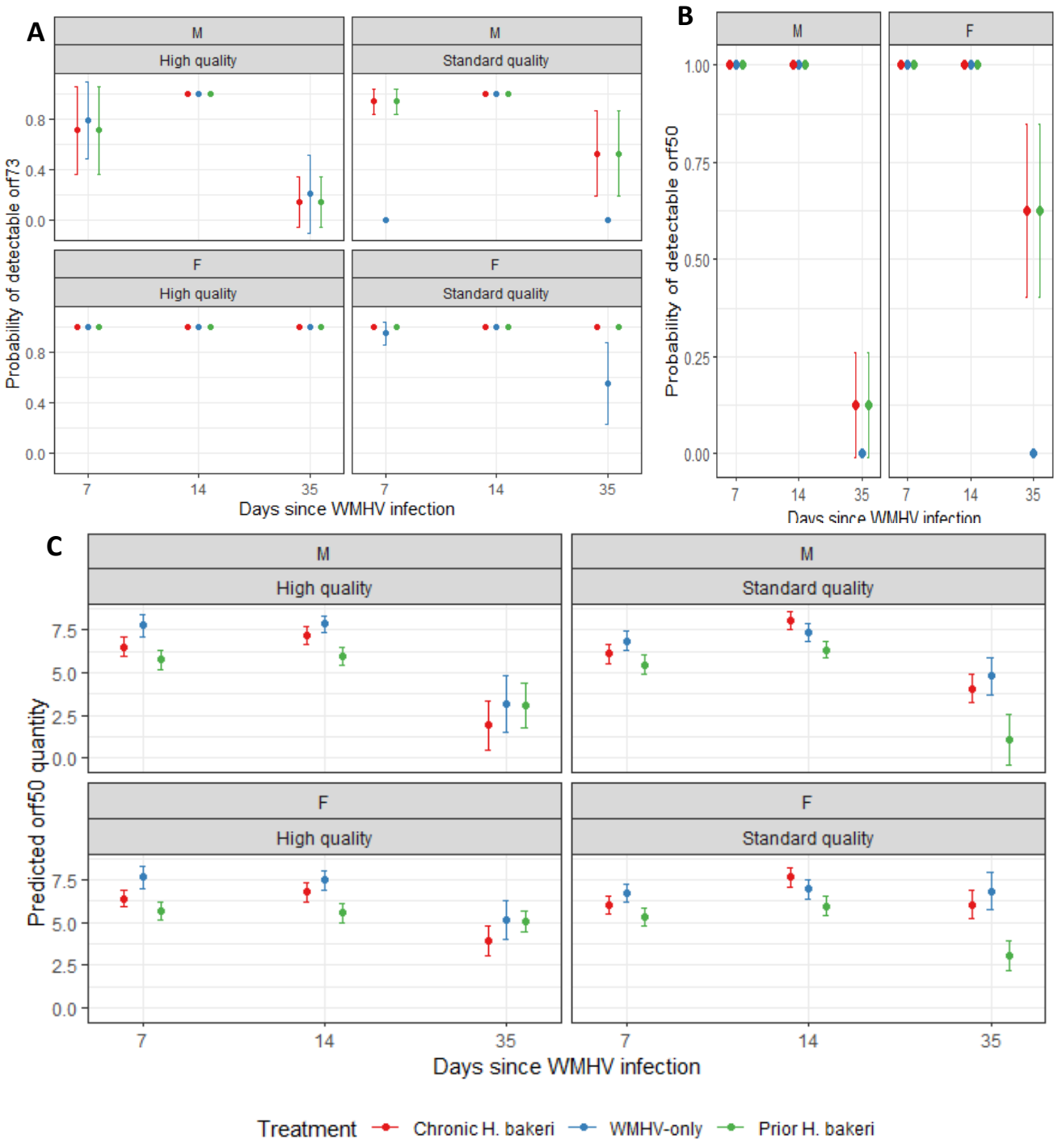

Figure 6.2. As sociation between a) probability of WMHV infection (detectable *ORF*73 expression) diet, sex and infection group , b) probability having an active WMHV infection (detectable *ORF*50), sex and infection group , c) predicted *ORF*50 quantity, diet, sex and infection group , and days since WMHV infection in the lungs of laboratory bread wood mice.

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